Assessment of the Antithrombotic Properties of Polar Lipids of Beer and Brewing Industry By-products.

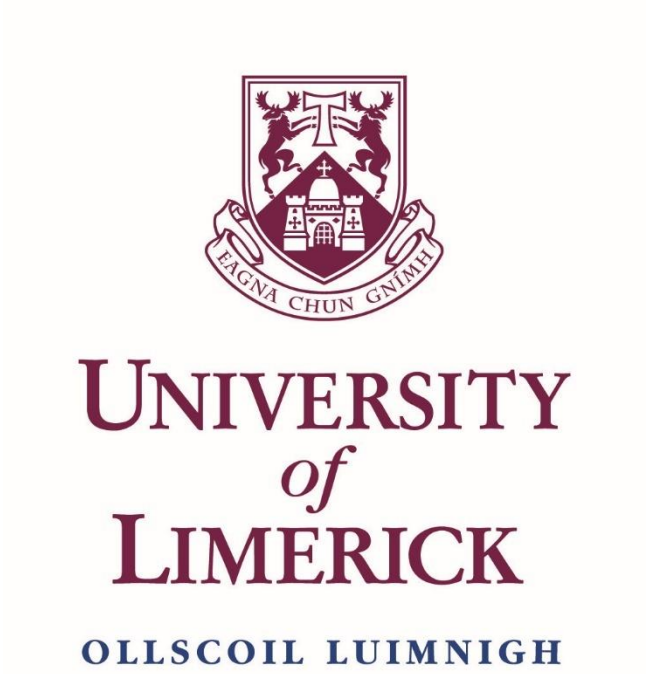

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Table of Contents

Abstract:

This study investigated the *in vitro* antithrombotic properties of the lipids extracted from malted grain (MG), brewer's spent grain (BSG), raw hops (RWHP), spent hops (SPHP), wort, and beer. Total lipids (TL) were extracted according to the Bligh & Dyer method and further separated into total polar lipid (TPL) and total neutral lipid (TNL) using a modified counter-current distribution method. The TPL of the beer was further fractionated by TLC and isolated bands were extracted. The antithrombotic activities of the TL, TPL and TNL of the MG, BSG, RWHP and SPHP were analysed *in vitro* for their ability to inhibit platelet-activating factor (PAF) induced aggregation in human platelets. Seven bands extracted by preparative TLC of the beer TPL were also tested for the ability to inhibit PAF induced platelet aggregation. This study demonstrated that the TPL extracts were the most bioactive with the exception of BSG as the TL displayed potent antithrombotic activity. The wort and beer TPL displayed the most potent inhibitory activity against PAF induced platelet aggregation. However, the beer TPL possessed the strongest inhibitory activity against PAF with the lowest IC_{50} value (7.8 ± 3.9 µg) and it was noted that fermentation of the wort increased the antithrombotic activity, which may warrant further investigation. The TLC bands 2 and 3 of the beer TPL exhibited potent inhibitory activity against PAF, which may be due to the presence of phenolic compounds. The fatty acid composition of the TPL extracts of all samples were analysed by gas chromatography-mass spectrometry (GC-MS). The GC-MS analysis of the TPL found that the wort and beer shared similar fatty acid compositions. The findings of this study provide new information into the effect of fermentation on the fatty acid composition and antithrombotic properties of beer. Further structural and functional studies are required to identify the exact compounds responsible for the observed biological activity of the TPL extracts.

Declaration

I hereby declare that this is my own work and it has not been submitted for the award of degree at any other university

Signed: _____

Acknowledgements

I would like to thank Dr Ioannis Zabetakis for the opportunity to advance my studies in the University of Limerick and for your support of my work through your advice and guidance. The time I spent working on this project has helped me to gain a wealth of knowledge in the areas of lipids, cardiovascular health, the brewing industry, and utilisation of by-products and is knowledge that I thoroughly enjoyed learning! A massive thank you to you for making it possible!

I would like to thank Ronan Lordan and Alexandros Tsoupras for your co-supervision, guidance and support throughout this project also. The skills I learned from you in the laboratory helped me to vastly improve my skills in a laboratory setting to a level where I am confident in my abilities. Your patience, guidance, help, positivity, and friendship will not be forgotten.

Finally, I would like to thank my family, friends, and girlfriend who provided their support, and in many cases their blood, to help support me through to the completion of this project.

List of Tables

List of Figures

List of Abbreviations

AA - Arachidonic acid

AC - Adenylate cyclase

ACS - Acute coronary syndrome

Akt - Protein kinase B

ATP - Adenosine triphosphoric acid

BSG - Brewer's spent grain

BSA - Bovine serum albumin

cAMP - Cyclic adenosine monophosphate

CDP - Cytidine diphosphate

CHD - Coronary heart disease

CL - Cardiolipin

CNS - Central nervous system

COX - Cyclooxygenase

$cPLA_2$ - Cytosolic phospholipase A_2

CVD - Cardiovascular disease

DAG - Diacylglycerol

DASH - Dietary approaches to stop hypertension

EBC - European brewing convention

eNOS - Endothelial nitric oxide synthase

ERK - Extracellular signal-regulated kinases

FAME – Fatty acid methyl ester

GC-MS – Gas chromatography-mass spectrometry

HPLC – High-performance liquid chromatography

IL - Interleukin

iNOS - Nitric oxide synthase

LDLc – Low-density lipoprotein cholesterol

L-PC- Lyso-phosphatidylcholine

LPCAT - Acetyl-CoA: lyso-PAF acetyltransferases

MAPK - Mitogen activated protein kinase

MG – Malted grain

MMP - Metalloproteinase

mTOR - Mechanistic target of rapamycin

NADPO - Nicotinamide-adenine dinucleotide phosphate oxidase

NF-kB - Nuclear factor-kappa light-chain-enhancer of activated B cells

PAF - Platelet-activating factor

PAF-R - Platelet-activating factor receptor

PC - Phosphatidylcholine

PE - Phosphatidylethanolamine

PI – Phosphatidylinositol

PI3K - phosphatidylinositol 3-kinase

PKC - protein kinase C

PKA - protein kinase A

PLC - phospholipase C

PPP – Platelet-poor plasma

PRP – Platelet-rich plasma

R_f – Retention factor

RNS - Reactive nitrogen species

ROS - Reactive oxygen species

RWHP – Raw hop

SD – Standard deviation

SM - Sphingomyelin

SPHP – Spent hop

TL – Total lipids

TLC – Thin-layer chromatography

TNF-α - tumour necrosis factor

TNL – total neutral lipids

TPL – Total polar lipids

UHPLC-MS/MS - ultra-high performance liquid chromatography-tandem mass spectrometry

VCAM^{-1} - Vascular cell adhesion molecule 1

VEGF = vascular endothelial growth facto

XO = xanthine oxidase

Chapter 1:

Literature review

1.1 Cardiovascular Disease

Cardiovascular disease (CVD) is a term used to describe a set of diseases that affect the heart and/or circulatory system. CVD is the number one cause of death worldwide, causing the deaths of 17.9 million people, of which 85% were attributed to heart attack and stroke (World Health Organisation 2017). In Europe, this number stands at 3.9 million people killed by CVD related diseases. In Ireland alone, it is estimated that 10,000 people die each year due to CVD (Irish Heart Foundation 2019). CVD is the leading cause of death in men in all but twelve countries and for women it is the leading cause of death in all but two countries in Europe (Wilkins *et al.* 2017). Due to the severity of this problem, research into the prevention of atherosclerosis and other precursors of CVD are a major objective of modern medical investigation.

The term cardiovascular disease covers a spectrum of heart complications such as coronary heart disease (CHD), ischaemic heart disease, stroke, peripheral artery disease, rheumatic heart disease, congenital heart disease, and heart failure. There is a broad range of CVD related illnesses that all relate to one another and the heart and circulatory system. There are numerous known risk factors for CVD, many of which are preventable. Despite this, CVD is still the number one cause of death globally (World Health Organisation 2017). While it is known that some risk factors for CVD cannot be prevented by an individual, such as a family history of heart disease or type I diabetes mellitus, many are known and avoidable. Many of these CVD risk factors include an unhealthy diet, lack of or insufficient exercise, smoking, and excessive consumption of alcohol.

1.2 Human and Economic Impact of Cardiovascular Disease

Developing countries have the highest rate of mortality with an estimated 82% of the total premature deaths caused by noncommunicable diseases and 37% of these deaths are

caused by CVDs. This is a trend that the World Health Organisation predicts will continue to rise. This will continue putting major pressure on health services around the world, as well as increasing financial pressure on individuals paying for treatment of CVD, many of whom struggle to afford it (World Health Organisation 2017).

In Ireland, CVD places a substantial economic strain on the Health Service Executive (HSE). A 2015 report into the cost of heart failure not only demonstrated the economic cost of heart failure to the individual/patient, the healthcare system, and society in Ireland, but it also demonstrated that these trends will continue to worsen unless preventative strategies and effective therapies are developed. The estimated cost of heart failure in Ireland amounts to approximately €660 million per year. These costs relate to the treatment of the initial event and hospitalisation, the after care of the patient, general practitioner appointments, clinical specialist appointments, and ambulance services (Irish Heart Foundation 2015).

1.3 Causes of Cardiovascular Disease

The chances of a person developing CVD can vary dramatically from one individual to the next depending on one's exposure to certain modifiable and unmodifiable risk factors. Having one or more of these risk factors does not indicate the development of CVD but it does give an indication as to the risk of developing these diseases (World Heart Federation 2017). Risk factors that are due to an individual's decision or lifestyle that can be changed to reduce their risk of developing the condition are classified as modifiable risks. These are characterised by a conscious decision(s) that an individual knows will lead to either a reduction of increased risk of developing CVD, these are discussed further in section 1.3.1 and 1.3.2.

1.3.1 **Unmodifiable Risk Factors**

Unmodifiable risk factors increase the chances of developing CVD based on some factors that are out of the control of the individual. Family history of heart disease, age, and ethnic background are three common non-modifiable risks.

The incidence of a cardiovascular event i.e. stroke, myocardial infarction, in a first-degree male relative such as a brother or father can dramatically increase that individual's chances of suffering from a cardiovascular event. The presence of heart disease amongst both parents can increase that person's chances of developing a CVD to over 50% in comparison to the general population (World Heart Federation 2017). One directly inheritable risk factor is familial hypercholesterolemia (FH) which is an inherited condition characterised by levels of low-density lipoprotein cholesterol (LDLc) that far exceed normal physiological levels, putting them at an increased risk for premature CVD (Sharifi *et al.* 2016). Premature CVD in men refers to a cardiovascular event <51 years and <56 years in women (Mulders *et al.* 2011).

Ageing is a natural and inevitable part of life and as we get older our risk of developing CVD or associated diseases increases. The average lifespan continues to rise and it is expected that by the year 2030 almost 20% of the world's population will be aged 65 and older, and within this age group, CVD is expected to be the main cause of death (North and Sinclair 2012). With an increase in population in the over 65 age bracket by 2030 and the associated increased risk of CVD, it is expected that the cost of treatment of CVD will also rise meaning that more awareness and preventative measures are required (Heidenreich *et al.* 2011).

1.3.2 **Modifiable Risk Factors**

Modifiable risk factors refer to the modifiable components in an individual's lifestyle that can impact their risk of developing CVD such as diet, behaviour, and lifestyle. In the battle against CVD, prevention is key and unlike the unmodifiable risk factors, measures can be taken to alter the lifestyle of an individual to lower the risk of CVD. Increasing physical activity, eliminating unhealthy behavioural patterns like smoking, and following a healthy dietary pattern as outlined below are examples of positive measures that can be taken to lower CVD risk.

Exercise is a pivotal modifiable risk factor for the prevention of many chronic diseases and physical inactivity can raise the incidence of up to 17 diseases, most of which are chronic diseases, including CVD (Booth *et al.* 2000). This demonstrates the importance of exercise as a point of primary prevention of numerous diseases. However, an investigation into the dietary and lifestyle habits of the Irish population, the SLÁN study, found that only 41% of Irish adults took part in moderate or strenuous physical activity for at least 20 minutes three or more times a week (Morgan *et al.* 2008). These statistics highlight the burden the Irish population put on themselves as it has been reported that even performing higher levels of moderate physical activity can reduce CHD related events by 21% in men and 29% in women (Li and Siegrist 2012).

Another prominent modifiable risk factor of CVD is smoking. It has long been known that smoking has been linked to many chronic diseases. The reason being that cigarette (tobacco) smoke affects smokers via multiple complex metabolic pathways involving exposure to free radicals from the components of tobacco smoke, leading to increased oxidative stress, inflammation, and DNA damage which can link smoking to many chronic diseases (Onor *et al.* 2017). Continued use of tobacco can dramatically reduce the overall health and wellbeing of an individual but eliminating tobacco use can improve an

individual's health by reducing their risk of a myocardial infarction by 65% dependent on other factors related the individual (Yusuf *et al.* 2004).

Along with the other modifiable risk factors, diet plays a critical role in CVD prevention. There is an ever-growing collection of data that supports the evidence demonstrating the pivotal role of nutrition in the development of numerous chronic diseases such as CVD. Diet and lifestyle are a key modifiable risk factors in the battle against CVD as can be seen when contrasting the dietary differences in parts of the world with the prevalence of CVD (Keys *et al.* 1984). Various dietary and environmental factors come into play when assessing CVD risk, however there is evidence to suggest that an individual's diet plays a significant role in determining their risk of developing CVD. In particular, comparison of individuals that abide by the DASH and Mediterranean diets compared to those that follow an unhealthy Westernised diet, indicates that diet can reduce one's risk of developing CVD. The DASH diet is a dietary pattern that targets the reduction of blood pressure, as high blood pressure increases one's risk of developing CHD, stroke, and cardiovascular events (Tyson *et al.* 2012). The original DASH diet was based on a low fat, less than 30% of total calories, and higher carbohydrate, ~55%, but has since reduced the percentage of energy for carbohydrates in exchange for additional vegetables and protein sources (Mozaffarian *et al.* 2011). The diet is characterised by low-fat dairy foods and reduced saturated fat intake that was found to significantly reduce blood pressure (Appel *et al.* 1997) and with lower rates of heart failure (Levitan *et al.* 2009). While the DASH diet has demonstrated relative success, the diet with the most evidence to support its role in preventing CVD is the Mediterranean diet (Tsoupras *et al.* 2018b). The Mediterranean diet consists of a high dietary intake of fruits, vegetables, wholegrain/wheat based cereal products, and monounsaturated fats. This is paired with fish, dairy, and alcohol consumption in moderation and a low intake of red and processed meats with more emphasis being placed on fish intake than other meats

(Tsoupras *et al.* 2018b). This diet has been the subject of numerous studies and has consistently shown a significant and consistent protection when the Mediterranean diet is adhered to against CVD and other chronic diseases (Sofi *et al.* 2010; Estruch et al. 2018).

1.4 Inflammation and Atherosclerosis

Inflammation is our body's natural defence mechanism and is a very complex response that is designed to protect us in the event of traumatic injury or against infectious organisms. It is a physiological process that is vital for the repair of tissue and the elimination of pathogenic insults (Tsoupras *et al.* 2018b). Inflammation is generally divided into acute inflammation, which is a response to tissue injury or a physical stressor, and chronic/systemic inflammation, the prolonged tissue reaction following the initial reaction that is often dominated by lymphocytes, plasma cells and macrophages. CVD is characterised by chronic inflammation as it takes place over a long period of time and the progression of CVD is influenced by its associated risk factors. This chronic inflammation is not only linked to CVD, but it is also an underlying feature of a variety of other chronic diseases, such certain cancers and type 2 diabetes, further emphasising the fact that understanding the development of systemic inflammation is key to the primary prevention of such diseases (Stokes and Granger 2012).

This inflammatory response aims to restore tissue homeostasis. This response is initiated by innate sensing mechanisms that detect the stressor such as infection by pathogens, stressed or dying cells releasing chemokines or a barrier breach in the endothelial cells of blood vessels. A cascade of inflammatory and mechanistic effects is well-orchestrated by the immune system in order to eradicate the causative agent (Tsoupras *et al.* 2018b). The immune system swiftly responds to any of these stressors with several immune cells being sent to/synthesized at the site of damage in an effort to remove the agent causing

the inflammatory response (Spinas *et al.* 2014). Should the removal of the stressor by the acute inflammatory response be successful the inflammatory response is terminated, and normal tissue function can resume. However, if the inflammatory response was unable to adequately repair the tissue injury, the inflammatory response becomes unresolved and systemic inflammation occurs leading to further tissue dysfunction (Tsoupras *et al.* 2018b). This can be associated with the development of an atherosclerotic plaque. Animal models have demonstrated systemic inflammation where lipid accumulation in the artery wall has occurred due to the presence of localised inflammatory markers such as blood leukocytes and platelet- activating factor (PAF), which are present in the early lesions of atherosclerotic plaque. This demonstrates the role inflammation plays in the onset and development of atherosclerosis (Li *et al.* 1993; Libby 2012).

Atherosclerosis is a chronic inflammatory disease resulting in the development of a plaque on the walls of medium and large arteries, occurring due to the failure of the inflammatory response system to remove the causative agent of the inflammation or repair the injured tissue. Atherosclerosis is characterised by the formation of plaques consisting of a necrotic core, calcified regions, accumulated modified lipids, inflamed smooth muscle cells, endothelial cells, leukocytes and foam cells (Ross and Dodet 1999). The presence of any of the risk factors associated with CVD can be attributed to the development of one of these atherosclerotic plaques. For example, toxins introduced into the bloodstream by smoking can cause damage to parts of the endothelial layer. Circulating in the blood, LDLc begins to bind to proteoglycans and migrate into the tunica intima at the site of damaged endothelial layer. The affinity of LDLc for the artery wall can be described by the attraction of positively charged amino acids from apolipoprotein B100 of LDLc interacting with the negatively charged sulphate or carboxylic acid groups from glycosaminoglycans (Flood *et al.* 2004). This affinity for the arterial wall allows LDLc to move into the intima in areas

where there is damage to the endothelial wall. Once inside the intima, the LDLc begins to oxidise, triggering an immune response as oxidised LDLc is toxic to endothelial cells. This lipid oxidation can cause the expression of adhesion molecules by damaged endothelial cells and the recruitment of chemokines, proinflammatory cytokines, and other mediators of inflammation to the affected region. These selective adhesion molecules allow for the recruitment of certain leukocytes such as macrophages in the earlier stages of atherosclerosis (Libby 2012). This endothelial activation and dysfunction results in the upregulating of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), P-selectin as well as monocyte chemotactic proteins such as CCL2 that facilitate the recruitment of monocytes (Angelovich *et al.* 2015).

Monocytes react to these adhesion molecules such as P-selectin, initiating the rolling of the monocyte along the endothelium until it comes into contact with VCAM-1, where rolling slows and the monocyte tightens to the endothelium (Galkina and Ley 2007). These monocytes migrate into the atherosclerotic prone region where they differentiate into macrophages for LDLc removal (Galkina and Ley 2009). The expression of scavenger proteins on the surface of these macrophages allows them to ingest lipids, as more LDLc migrates to the intima. These macrophages eventually reach capacity and differentiate into foam cells and as these started as monocytes, the death of these cells triggers the release of cytokines (Libby 2012). The production and release of proinflammatory cytokines and chemokines such as tumour necrosis factor and macrophage inflammatory protein-1 drives the inflammation further as the atherosclerotic plaque grows (Wong *et al.* 2012). As the inflammatory response intensifies, activated leukocytes and other intrinsic arterial cells release fibrogenic mediators such as cytokines that promote the replication of smooth muscle cells of the tunica media and their migration into the atherosclerotic plaque (Galkina and Ley 2009). These cells are responsible for the formation of connective tissue, and in

atherosclerotic lesions these tissues are fibrous and form around the plaque that overlies the necrotic core shielding it from the bloodstream (Ross and Dodet 1999). However, the death of these smooth muscle cells over time further hinders the arteries, as apoptosis of smooth muscle cells promotes the calcification of the matrix causing stiffening of the arteries (Durham *et al.* 2018). Eventual degradation of the fibrous cap by mechanical stress from the blood-flow through the occluding artery or enzymes secreted by macrophages that are capable of breaking down the extracellular matrix can reduce the cap strength (Arroyo and Lee 1999). Weakening of the fibrous cap can leave it prone to rupture. Upon rupture, it can expose the thrombogenic lipid core, red-cell rich necrotic core material within the cap to the bloodstream resulting in coagulation and thrombosis within the affected artery, potentially causing complete occlusion of the artery resulting in a cardiovascular event such as myocardial infarction or ischemic stroke (Bentzon *et al.* 2014).

1.5 The Role of Platelet-Activating Factor

Platelet-activating factor (PAF) is a potent proinflammatory mediator, the function of which under normal and inflammatory conditions has been the subject of research for many years. The term PAF was originally used in 1972 by Benveniste *et al* in his research, revealing that it is released from rabbit basophils in an Immunoglobulin E-dependent process (Benveniste *et al.* 1972). Not much was known of PAF structure or function at this point until two independent studies were able to describe the structure of PAF as 1-*O*-alkyl-2-acetyl-*sn*-glycero-phosphocholine, with the classic model being an alkyl ether linkage at position *sn*-1, acetyl group at position *sn*-2 and a phosphocholine group at position *sn*-3 as seen in Figure 1.1 (Benveniste *et al.* 1979; Demopoulos *et al.* 1979). Since its discovery, it has been associated with a number of roles within the body under normal conditions but also as a vital mediator during inflammatory conditions within the body, being synthesised by

several cells upon activation such as platelets, monocytes, macrophages, foam cells as well as endothelial cells, further highlighting the role it plays in atherosclerosis (Demopoulos *et al.* 2003), which will be further discussed.

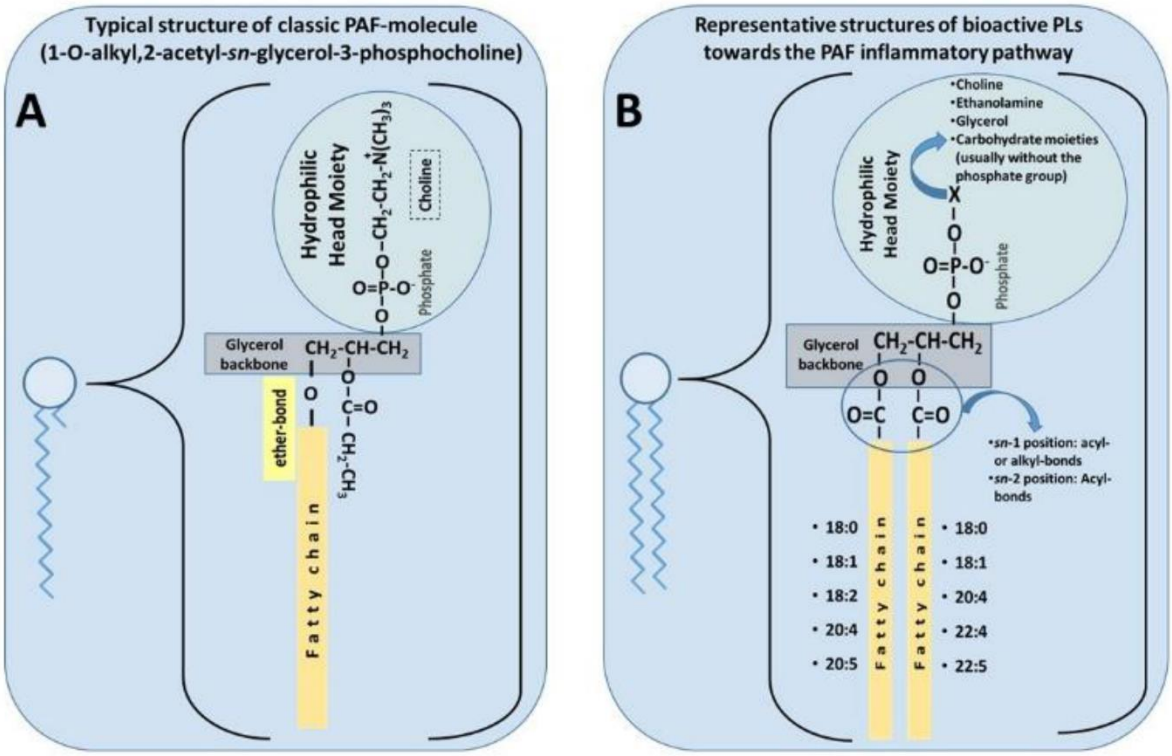

Figure 1.1. (A) Typical structure of a PAF molecule. (B) Image representing a biologically active phospholipid that mimics PAF.
Reproduced with permission from (Lordan *et al.* 2018a).

The synthesis of PAF within the body is tightly regulated due to its potency and role in a diverse range of functions. Two distinct pathways are known to exist depending on the requirements of the body. The first of these pathways, the *'de novo'* pathway, is responsible for the basal levels of PAF. PAF synthesised by this route, often in the brain and kidneys, serves a role primarily in homeostasis, maintaining the PAF levels throughout the body for normal cellular functions (Palur Ramakrishnan *et al.* 2017). PAF is synthesised from the

substrates alkylacetylglycerol and Cytidine diphosphate (CDP)-choline. The initial step of the *'de novo'* pathway is the acetylation of alkylglycerol-P by the catalytic action of the enzyme acetyltransferase to form alkylacetylglycerol-P before dephosphorylation by a specific phosphohydrolase reduces alkylacetylglycerol-P to alkylacetylglycerol, one of the two primary substrates in the formation of PAF. The final step is catalysed by diacylglycerol cholinephosphotransferase (EC 2.7.8.16) that utilises the aforementioned alkylacetylglycerol and CDP-choline as co-substrates to form PAF via the *'de novo'* pathway (Snyder 1997).

The alternative pathway by which PAF is produced is the remodelling pathway, that tends to be associated more with the acute proinflammatory production of PAF under activation of several cells during inflammatory conditions (Tsoupras *et al.* 2018b). By this pathway, PAF is synthesised by many cells in response to specific stimuli including cytokines, endotoxins, Ca^{2+} ionophores, and also in response to PAF itself, highlighting why it is be believed that PAF is a common mediator among many chronic inflammatory diseases (Lordan *et al.* 2019b). During inflammatory conditions, the ether analogue of phosphatidylcholine (PC) is converted to lyso-PAF by the action of phospholipase A_2. This lyso-PAF, an inactive form of PAF, is then acetylated by isoforms of acetyl-CoA and lyso-PAF transferases to yield the biologically active form of PAF (Lordan *et al.* 2019b).

PAF is synthesised by several cells (e.g. macrophages, foam cells, endothelial cells, and platelets) involved in the inflammatory process of atherosclerosis once they are activated. PAF and PAF-like lipids act through their high affinity binding to a unique G-protein coupled seven transmembrane receptors called a PAF-receptor (PAF-R), expressed on the surface of various cells (Honda *et al.* 2002). PAF or PAF like molecules upon binding to the PAF-R activates multiple intracellular pathways in blood or tissue cells bearing the PAF-R and can cause a cascade of intracellular responses that in many cases initiate or

amplify inflammatory processes (Yost *et al.* 2010). The role of PAF in the inflammatory process of atherosclerosis has been reported in studies from initiation and recruitment of inflammatory cells to and across the endothelium by promoting the surface expression of adhesion molecules, right up to the rupture of the fibrous cap at which point platelets activated during binding to the ruptured endothelium release PAF and subsequent overexpression of PAF to the site of rupture, resulting in continuous recruitment, aggregation and subsequent thrombosis (Zimmerman *et al.* 2002; Palur Ramakrishnan *et al.* 2017).

Without specific binding to the PAF-R, PAF synthesis and activation capabilities are lowered, demonstrating that potential therapeutic approaches to these inflammatory actions should focus on the PAF-R and its interactions, inhibiting the exacerbation of the PAF-induced response model through competitive and non-competitive displacement of PAF from the PAF-R by PAF-R antagonists (Lordan *et al.* 2018b). PAF-R antagonists that act in this way are often polar lipid extracts from foods associated with that of the Mediterranean diet such as olive oil, wine, dairy, and fish, all of which have shown promising results due to the polar lipids ability to act on the PAF-R (Karantonis *et al.* 2006; Tsantila *et al.* 2007; Tsoupras *et al.* 2018b).

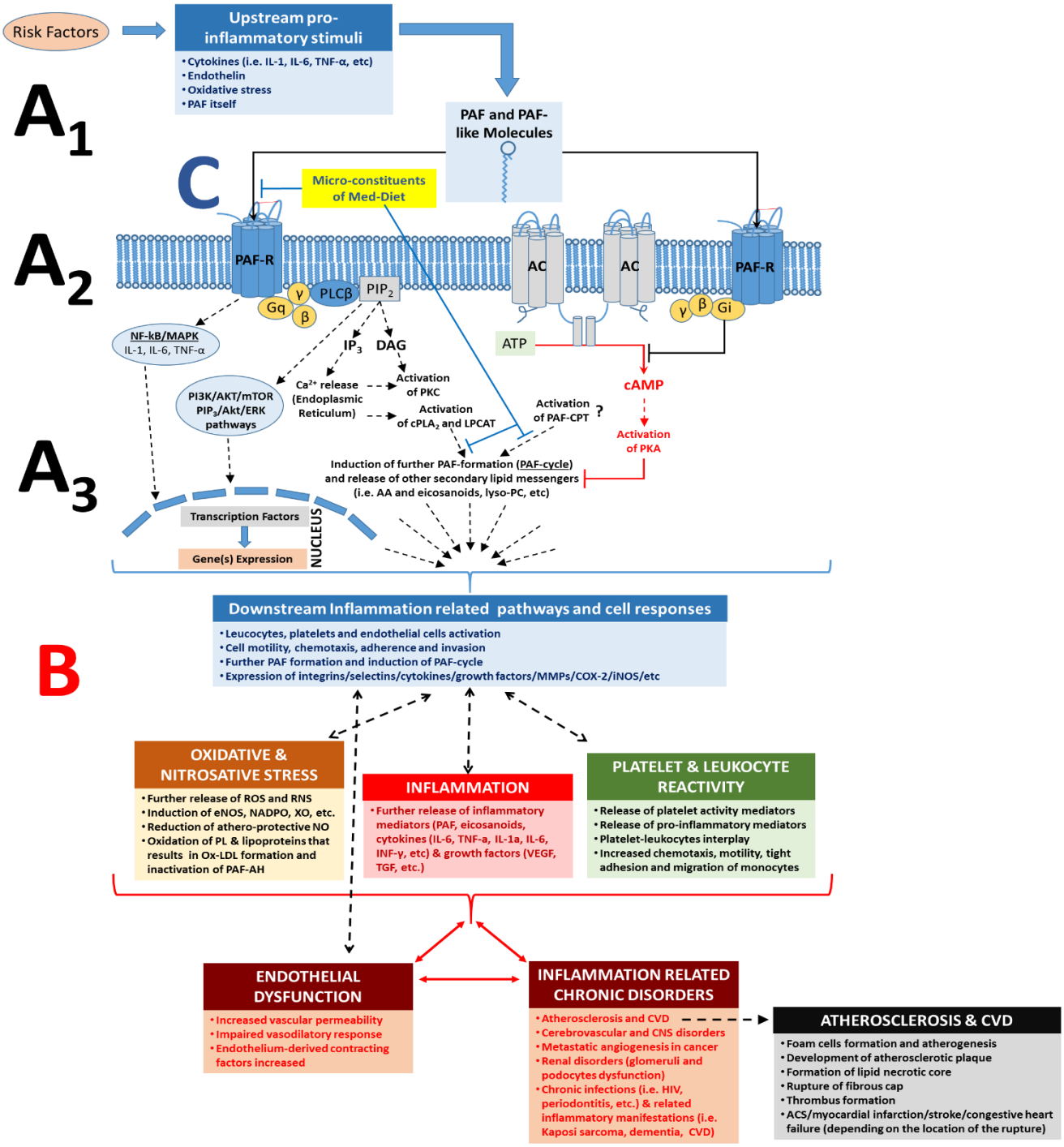

Figure 1.2. An image demonstrating the role of PAF and PAF-R in response to inflammatory stimuli.

(A1) Pro-inflammatory stimuli stimulate the syntheses of PAF and the expression of PAF-R on the surface of cells which can result in the amplification of certain pro-inflammatory stimuli. (A2) The binding of PAF to PAF-R signals the initiation of several inflammatory responses such as the release of PAF from the activated cells and the production of more arachidonic acid within the body to be used as a substrate in the formation of more PAF. (A3) Further activation of PAF-R on the surface of cells signals the release of chemokines, expression of adhesion molecules on endothelial cells, and release/synthesis of PAF, further amplifying the inflammatory response. This results in the cellular signalling for the

recruitment of lymphocytes to the site of inflammation where lymphatic cells bind to the adhesion molecules expressed on the surface of the damaged endothelial cells. (B) The inflammatory cascade leads to an increase in the concentration of PAF at the site of inflammation and so promotes a broad spectrum of effects caused by the increased levels of PAF and other pro-inflammatory mediators circulating the blood. The increased levels of PAF, chemokines, and other inflammatory mediators in the bloodstream causes further activation of PAF-R. This promotes the aggregation of platelets and leukocytes and subsequent damage and activation of other endothelial cells downstream. These damaged endothelial cells promote leukocyte adherence, invasion, migration and subsequent endothelial dysfunction, thus stimulating the development of an inflammatory-related chronic disorders as seen in this image. (C) Demonstrates how PAF antagonists, such as microconstituents of the Mediterranean diet, can act to disrupt the binding of PAF to the PAF-R. This disruption of PAF, possibly occurs by competitive displacement and, could aid in lowering PAF concentrations in the blood (Image reproduced with permission from Tsoupras 2018b).

Abbreviations: AA = arachidonic acid; AC = adenylate cyclase; ACS = acute coronary syndrome; Akt = protein kinase B; ATP = adenosine triphosphoric acid; cAMP = cyclic adenosine monophosphate; CNS = central nervous system.; COX: cyclooxygenase; $cPLA_2$ = cytosolic phospholipase A_2; CVD = cardiovascular diseases; DAG = diacylglycerol; eNOS = endothelial nitric oxide synthase; ERK = extracellular signal-regulated kinases; IL = interleukin; iNOS = nitric oxide synthase; LPCAT = acetyl-CoA: lyso-PAF acetyltransferases ; MAPK = mitogen activated protein kinase; MMP = metalloproteinase; mTOR = mechanistic target of rapamycin; NADPO = nicotinamide-adenine dinucleotide phosphate oxidase; NF-kB = nuclear factor-kappa light-chain-enhancer of activated B cells; TNF-α = tumour necrosis factor; PAF = platelet activating factor; PAF-R = Platelet-activating factor receptor; PI3K: phosphatidylinositol 3-kinase; PKC = protein kinase C; PKA = protein kinase A; PLC = phospholipase C; RNS = reactive nitrogen species; ROS = reactive oxygen species; VEGF = vascular endothelial growth factor; XO = xanthine oxidase;

1.6 Polar Lipids

Lipids are a heterogenic class of biomolecules with wide ranging structures and functions. The two major sub-classes of lipids are neutral lipids and polar lipids. Neutral lipids, such as triacylglycerols, waxes, and terpenes, consist of hydrophobic, hydrocarbon chains bound to a glycerol backbone. The primary difference between neutral lipids and polar lipids is the absence of a charged phosphate group in the neutral lipids. Polar lipids, such as phospholipids and glycolipids, also contain the hydrophobic hydrocarbon tails bound to a glycerol, accompanied by a carbohydrate-group or phosphate-head group with a hydrophilic residue within their structure (Lordan *et al.* 2017). These phospholipids can be

found in all plant and animal cell membranes. Their amphipathic properties allow them to form a lipid bilayer, most of these cell membranes being made up of glycerophospholipids (Küllenberg *et al.* 2012). These glycerophospholipids share the common structure of two fatty acid molecules esterified to the glycerol backbone in the *sn*-1 and *sn*-2 positions of the molecule contributing to the hydrophobicity of the molecule while a phosphate group at the *sn*-3 position gives the molecule its hydrophilicity. This is known as phosphatic acid and is the simplest glycerophospholipid, with others being named in accordance with the molecule that attaches to the phosphate group. The main groups are ethanolamine, inositol, serine and choline that yield phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and (PC) respectively (Castro-Gómez *et al.* 2015)

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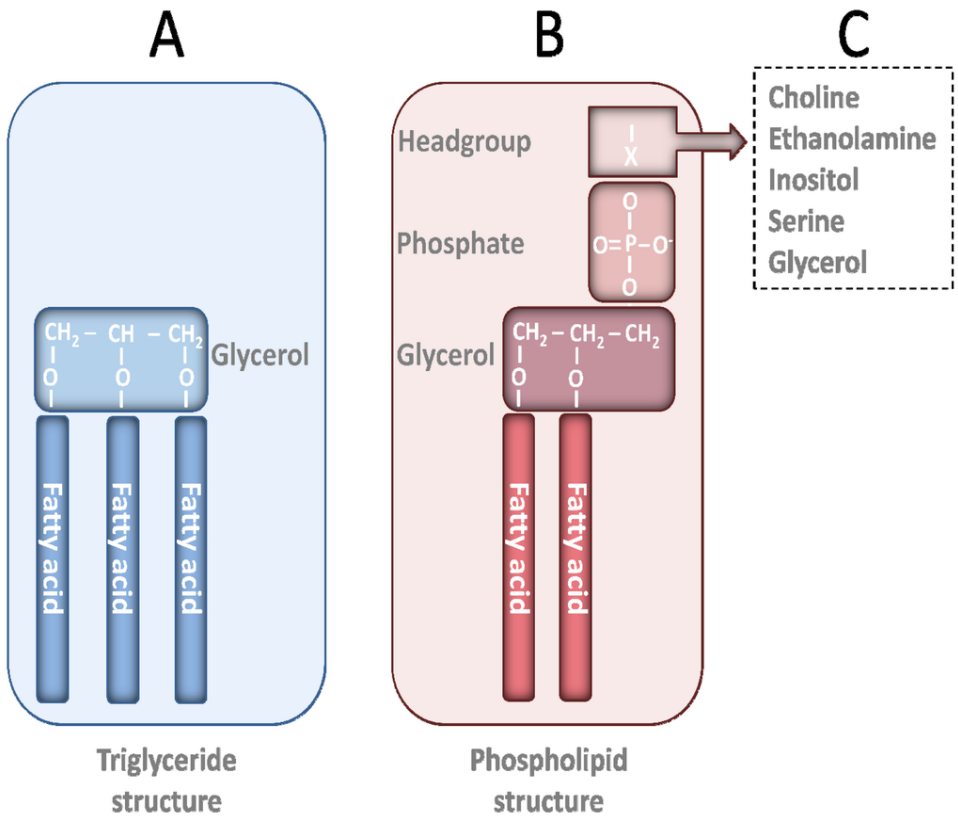

Figure 1.3 (A) Structural representation of a triglyceride molecule. (B) Structural representation of a polar lipid molecule.

The polar lipid structure bears two hydrophobic, hydrocarbon chains esterified to the glycerol backbone in the *sn*-1 and *sn*-2 position, with a charged phosphate group at the *sn*-3 position. (C) The headgroup that attaches to the phosphate molecule and gives the molecule its hydrophilic properties (Imaged reproduced with permission from Burri *et al* 2012)

The integrity of the phospholipid bilayer is essential to mammalian life; no membranes or cells are formed without these. The bilayer is formed in accordance with the amphiphilic nature of the lipid with hydrophobic hydrocarbon tail orientated to the interior of the cell membrane, while the hydrophilic polar group orientate toward the outer surface of the cell membrane in order to interact with the water phase providing a low-permeability to certain cellular constituents such as nutrients or ions while the formation of this lipid matrix supports the diverse functions of the cell (Caforio and Driessen 2017).

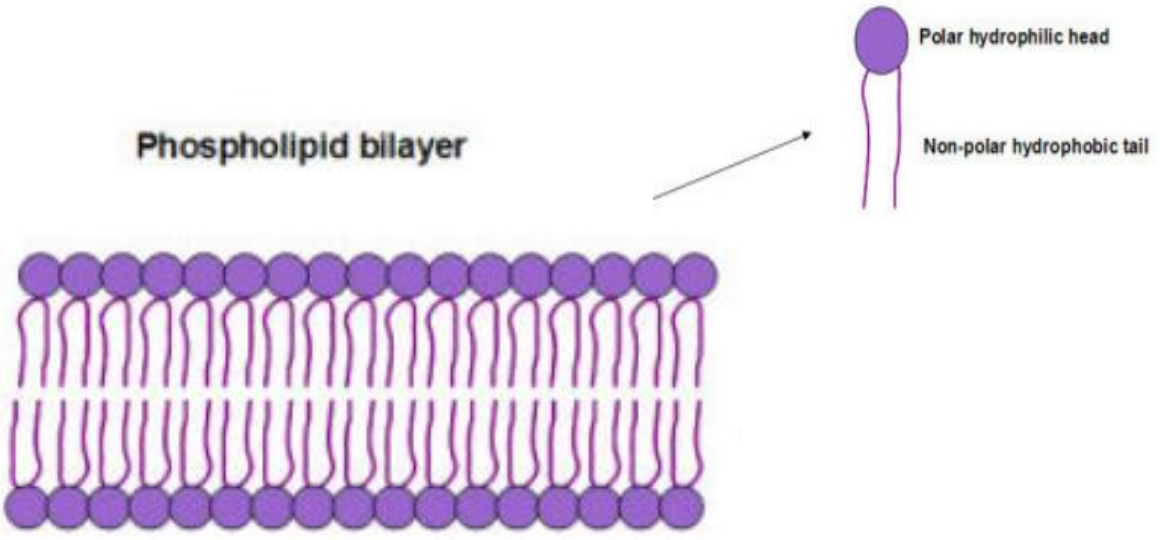

Figure 1.4. Image representation of the phospholipid bilayer.

The Image displays how polar lipids are orientated in a phospholipid bilayer, with the polar hydrophilic head having a greater affinity for the aqueous phase. The hydrocarbon tails are highly hydrophobic, so lipid molecules orientate as displayed in this image to avoid interaction with the aqueous phase. This bilayer is essential for mammalian life as the formation of membranes or cells cannot be formed without it. (Image reproduced with permission from Belhocine and Prato 2011).

These polar lipids not only play a role in cellular functions but dietary polar lipids from various food sources have displayed anti-inflammatory properties against PAF. These anti-inflammatory properties have been attributed to polar lipids ability to interrupt the action of PAF on PAF-R through competitive and non-competitive displacement of PAF from the PAF-R, and through the modulation of PAF metabolic enzymes toward homeopathic PAF levels (Nasopoulou *et al.* 2011; Lordan *et al.* 2019c). The anti-PAF potential of lipids from various dietary sources has been investigated, with evidence suggesting that the most potent anti-inflammatory polar lipids were found in foods associated with the Mediterranean diet such as fish, olive oil, wine, and dairy products (Fragopoulou *et al.* 2001; Tsantila *et al.* 2007; Nasopoulou *et al.* 2011; Lordan and Zabetakis 2017).

The association of the Mediterranean diet with a lower incidence of CVD, paired with the fact that the most anti-inflammatory and antithrombotic polar lipids can be found within defining aspects of the diet has led research to delve deeper into this association. Studies have been published that demonstrate the anti-inflammatory properties of olive oil, olive pomace (Tsantila *et al.* 2007), and marine lipid sources (Nasopoulou *et al.* 2010). A separate *in vivo* study demonstrated that the use of olive pomace in fish feeds improves the nutritional value of the fish feed and the fish with improvements to the lipid profile of the fish demonstrating the effective implementation and use for valorising this by-product (Nasopoulou *et al.* 2013b).

The consumption of dairy products has long been negatively associated with cardiovascular health due to the high saturated fat content of dairy foods and the association of saturated fats with raised cholesterol levels. This has resulted in a rise in the consumption

of low-fat or fat free dairy products in an effort to adhere to dietary guidelines, but evidence on the health benefits of dairy products has started to change this perception, with various dairy foods exhibiting cardioprotective and anti-inflammatory effects (Dugan *et al.* 2016; Poutzalis *et al.* 2016; Lordan and Zabetakis 2017). Another aspect of dairy products that is being explored is the impact of fermentation on the anti-inflammatory properties of cow, goat, and sheep produced yogurts with a study by Lordan *et al* (2019) demonstrating that specific starter cultures can alter the fatty acid composition of the polar lipids and antithrombotic properties (Antonopoulou *et al.* 1996; Tsorotioti *et al.* 2014; Lordan *et al.* 2019c).

1.6.1 Polar Lipids and Alcoholic Beverages.

If fermentation can alter the antithrombotic capabilities of polar lipids in dairy products, then the same could possibly be said for fermented beverages as most alcoholic beverages are fermented with yeast that have the capability to alter the lipid present in beer (Taylor and Kirsop 1977; Buiatti 2008). Another aspect of the Mediterranean diet that differs from the western diet is the moderate consumption of wine, predominately red wine, with meals. Though many negative connotations surround alcohol due to diseases associated with its abuse, studies have demonstrated that when consumed in moderation there can be cardioprotective effects (Argyrou *et al.* 2017; Xanthopoulou *et al.* 2017).

Research into the cardioprotective properties of wine increased when the term ‘French Paradox’ was used to describe the observation that there was a relatively low-incidence of CHD among the French population despite their diets being rich in saturated fats, which is considered a major risk factor for CVD. The authors suggested that this may be attributed to relatively high level of wine intake among the population, an observation

similar to that of the Mediterranean diet (Renaud and de Lorgeril 1992). Though the 'French Paradox' has not been established as a cause/effect relationship, this observation motivated research into the relationship between moderate wine consumption and potential cardioprotective effects in relation to CHD. Many studies support the moderate consumption of wine for its cardioprotective effects due to its microconstituents and ethanol content (Fragopoulou *et al.* 2018; Lordan *et al.* 2018a). While it is known that excessive consumption of alcohol has been linked to a number of chronic diseases (Temple 2012), recent research has indicated that alcohol can have a positive impact on cardiovascular health when consumed in moderation (20-30g of ethanol for men and 15-20g of ethanol for women), potentially displaying a causal relationship between CHD risk and moderate alcohol consumption could be the explanation for this (Mathews *et al.* 2015). The relationship between moderate alcohol consumption and reduced CVD risk and all-cause mortality is characterised by a J-shaped curve as seen in Figure 1.5 (de Gaetano *et al.* 2016). Apart from lipids, other microconstituents found in wine have been associated with cardioprotective effects, such as the inhibitory effects of polyphenols, tyrosol and resveratrol, have been shown to exert an inhibitory effect on PAF production and platelet aggregation (Vlachogianni *et al.* 2015b) and anti-oxidant activity (Vlachogianni *et al.* 2015a).

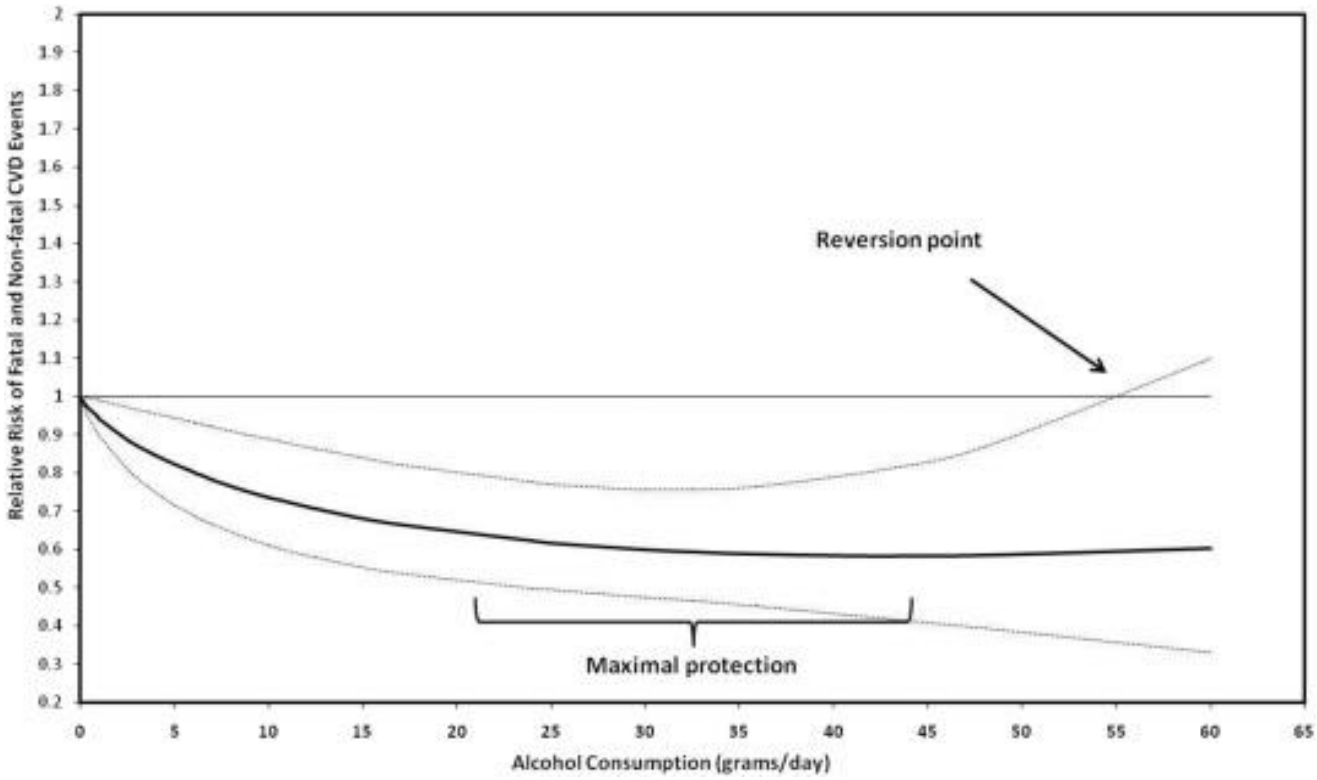

Figure 1.5. A graph that demonstrates the relationship between alcohol intake and relative risk of non-fatal cardiovascular events.

This relationship is characterised by a J-shaped or U-shaped curve (Image reproduced with permission from de Gaetano *et al* 2016)

The method that is regarded as standard for the analysis of the effect of lipids against platelet aggregation is by light transmission aggregometry in platelet-rich plasma (PRP), in which lipid fractions, namely the total lipid (TL), total polar lipids (TPL), and total neutral lipid (TNL) fractions are analysed for their ability to inhibit or induce platelet aggregation in the presence or absence of an agonist such as PAF or thrombin (Tsoupras *et al.* 2019b). Many of these antiplatelet compounds have been found in wine. TL, TPL TNL of wine and wine must, which is the unfermented and unfiltered crushed grape juice, were assessed for their ability to inhibit PAF-induced platelet aggregation. It was observed that the biological activity of the TL and TPL lipid fractions there are more potent in their ability to inhibit PAF-induced platelet aggregation, while the TNL do not seem to exhibit strong biological

activity. The biological activity of the TL was attributed to the actions of the TPL within the TL present (Fragopoulou *et al.* 2001).

A study conducted by Fragopoulou *et al* (2000) obtained the TPL from Greek red wine and further separated the TPL into glycolipids and phospholipids by high-performance liquid chromatography (HPLC). Each fraction was tested for their ability to induce or inhibit PAF-induced platelet aggregation. The findings of this study demonstrated the existence of compounds within the TPL fractions of red wine, some of which exhibit PAF inhibition while others antagonise PAF via interactions with the PAF-R (Fragopoulou *et al.* 2000). The use of HPLC analyses in this study allowed a greater insight into the biologically active fractions of lipids, with the HPLC yielding fractions of PC, sphingomyelin (SM), lyso-phosphatidylcholine (L-PC), and PE alongside other glycolipids with all fractions demonstrating some degree of biological activity (Fragopoulou *et al.* 2000). The presence of many of these anti-inflammatory polar lipids have been found in other sources such as fish (Tsoupras *et al.* 2019a), and other fermented beverages (Lordan *et al.* 2019a).

Though the antithrombotic properties of another fermented product, beer, has not been widely reported and it is an area that requires further investigation. It has been demonstrated that wine and its by-products such as must contain polar lipids and various microconstituents that exhibit potent antithrombotic properties. Therefore, beer and various by-products may also possess these bioactive lipids. A previous investigation into the biological properties of the TL, TPL, and TNL of 3 Irish beers, stout, ale, and lager, revealed that the TPL fraction of all three Irish beers demonstrated potent antithrombotic properties *in vitro* further suggesting that fermentation may play a role in the formation of these biologically active lipids (Lordan *et al.* 2019a). This study also went on to separate the TPL by thin-layer chromatography (TLC) and found polar lipid fractions like those found in other anti-inflammatory foods like wine, demonstrating the presence of PE, PC, cardiolipin (CL), and

SM. These fractions were tested *in vitro* against PAF-inducing aggregation and sphingomyelin, PE, and CL demonstrated promising anti-PAF activities (Lordan *et al.* 2019a).

There is an ever-increasing body of evidence for the associated health benefits of wine consumption with very little attention being given to beer. Much of the research conducted regarding wine seems to attribute these effects to the effect of ethanol on the lipid profile or microconstituents present within the wine, but as demonstrated by Lordan *et al* (2019a) some microconstituents present in wine were also present in the three different beer types that demonstrated potent inhibition of PAF-induced platelet aggregation. More research is required to assess the bioactivities of beer, its microconstituents, and potential valorisation of by-products with regards to cardiovascular health.

1.7 Brewing and Beer Composition

The initial brewing of beer has been estimated to extend as far back as 8000 years, but it is only with advancements in the underlying science being made over the past 150 years or so that brings us to the modernised practice of brewing we are now familiar with (Bamforth 2000).

Ale, stout, and lager are three types of the most popular and common types of beer brewed all over the world. These are made using four key ingredients: malted grain (*Hordeum vulgare*), water, hops (*Humulus lupulus***),** and yeast and by varying the temperature in the system, yeast strain, grain roasting time and method and time of the hop addition can result in many unique types of beer. There are many steps where these modifications can take place, however, the process remains relatively consistent following the pattern of malting, milling, mashing, mash filtration, boiling, fermentation, and packaging.

Malting is a three-step process and is key to the quality of the fermentation and of the beer. This step involves taking harvested grain, be that barley or wheat, and making it suitable for the brewing process. This three-step process involves steeping, germination and drying. The objective of malting is to increase the water content from ~12% moisture content to between 43%-46% moisture content to promote the controlled germination of the barley grain (Brookes *et al.* 1976). During this germination, hydrolytic enzymes are activated by the grain that degrade the cell walls and protein, softening the husks for milling while subsequently producing the enzymes that will degrade the starch into fermentable sugars for the yeast. The final step in malting is kiln drying, which is a heating process designed to halt the metabolism of the grain to prevent the germination consuming the starch reserves by removing water and making it stable for storage, but also for the purpose of roasting the grain to attain a desired flavour, colour, and aroma (Bamforth and American Society of Brewing 2006).

The process of malting makes the grain suitable for the brewing process. The next step of the process includes milling and mashing. Milling involves the crushing of the modified grain into course, flour like powder known as 'grist'. This process exposes the modified starch reserves and good quality milling allows maximum surface area to contact with water which occurs in mashing. The grist is added to a mash tun in the presence of water to dissolve the solid matter. The mashing step generally begins at lower temperatures allowing more heat sensitive enzymes to continue the enzymatic conversion of polymers before the temperature is raised to approximately 65℃ where starch-degrading enzymes such as α-amylase convert starch to simple, fermentable sugars while proteolytic enzymes degrade proteins and peptides into amino acids to facilitate the growth of the yeast during fermentation (Briggs 1962; Bamforth 2014). From this, the sugar rich liquid is separated by filtration or lautering to yield the 'sweet wort'.

The sweet wort is boiled to facilitate the removal of undesirable organisms and to concentrate the wort before fermentation. Hops are added to the wort during boiling for the addition of flavour and aroma by compounds that are released from the hops as they boil. As the hops boil, the isomerisation of resins leads to the formation of bitter compounds, which are essential to the development of beer flavour, but might also contribute to the presence of various phytochemicals (Bamforth 2014). Following filtration and cooling, this liquid is transferred to a fermenter for pitching with the yeast. The appropriate yeast is selected for the target output. *Saccharomyces cerevisiae* is known as the ale yeast with fermentation occurring at as high as 25°C and *Saccharomyces pastorianus,* the lager yeast, fermenting at temperatures as low as 6°C (Bamforth 2000). After the required timeframe the pitched yeast will have fermented the maltose and other sugars to ethanol and carbon dioxide while also producing other molecules that affect flavour under primarily anaerobic conditions (Bokulich and Bamforth 2013). The beer is then subjected to cooling, or cold conditioning, for up to 3 days followed by filtration to facilitate the precipitation and removal of solids before being packaged into the desired container and shipped to the consumer (Bamforth 2000).

Though the core ingredients remain the same, beer all over the world can vary due to the variety of malted grain selected, the temperature used, yeast strain, pH throughout the brewing process, the variety, and more importantly method of hop addition, can all have an impact on quality and composition of the end product yielding several hundreds of identified compounds that contribute to flavour, aroma, texture, foam stability, and nutritional value of beers (Gerhäuser 2005; Buiatti 2008). Some of these compounds originate in the raw materials like the malt and hops while some are the result of yeast metabolism of the fermentable sugars. As mentioned it is subject to variations, but the general composition of beer is water, ethanol, carbon dioxide, carbohydrates, inorganic salts, organic acids, hop

derivatives, and some B vitamins (Buiatti 2008). The majority of the mineral content of beer come from the natural ingredients, with minerals such as calcium, magnesium, potassium, sodium, copper, zinc, and manganese being identified in beer samples with many of these B-vitamins being crucial to the growth of the yeast originating in the embryo and aleurone layer of malted grain (MG) (Muy-Rangel *et al.* 2018). Other components found in beer such as phenolic compounds, esters, aldehydes, ketones, and sulphur compounds also contribute in different ways to affect flavours and aromas but too high a level of some of these compounds as a result of reactions during the brewing process can cause various undesirable end-products (Buiatti 2008). These phenolic compounds are abundant in the human diet from sources such as plants, tea, fruits, and vegetables and as discussed, red wine. In beer, several polyphenols are derived from the malt (two-thirds) and the hops (one-third) (Buiatti 2008). As discussed, these polyphenols, such as resveratrol and anthocyanins in wine and xanthohumol and its metabolites in beer, could be potential sources of molecules that provide cardiovascular protection (Arranz *et al.* 2012).

The malted barley is the primary source of the protein, lipids, carbonate, and polyphenol compounds in beer. The protein is degraded to peptides and amino acids for use by the yeast during fermentation, but some remain in the final product, while the lipid content that is also obtained from the malted barley is lost mainly in the spent grain with a very low amount retaining in the final product (Buiatti 2008). However, it has been demonstrated that lipids extracted from the malt present in wort undergoes modification by the action of yeast during fermentation. Medium chain length fatty acids were present in low concentrations in the wort, but it was observed that during fermentation, short chain fatty acids were catabolised by yeast, which then produced some medium chain length fatty acids, formed by a synthetic route as opposed to the degradation of long chain fatty acids (Taylor and Kirsop 1977). This is reflected in the GC-MS analysis of three Irish beers; stout, ale, and lager, that found a

higher percentage of medium to long chain fatty acid such as 14:1, 16:0, 18:0, 18:1 and 18:2 in the polar lipid fatty acid composition (Lordan *et al.* 2019a)

The concentration of lipid present in any beer is dependent on a few variables such as the variety of barley grown (Bravi *et al.* 2012), the malting conditions, and the mash temperature (Evans *et al.* 2013). The lipid content of barley is approximately 3-4% at the beginning but during the malting process there is a loss of lipids during germination due to the hydrolysis of some of these triglycerides to free fatty acids (Anness 1984). Once malted, ~80% of the fatty acids still present are either neutral lipids or free fatty acids, while phospholipids and glycolipids make up the remainder (Anness 1984). These phospholipids have been identified as PC, PI, and L-PC, where PC and PE were identified as two major classes (Hough *et al.* 1982; Anness 1984).

The highlighted variables such as growing type and mash conditions makes the fatty acid composition of the malt barley, wort, and beer vary in the literature, though the dominant fatty acids in the malt, wort, and beer tend to be palmitic acid (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) (Bravi *et al.* 2012; Gordon *et al.* 2018). Bravi et al (2012) observed that mashing at temperatures over 65°C can increase the concentration of linoleic and linolenic as the higher temperature allows these fatty acids to be absorbed from the aleurone layer of the barley that is often not absorbed at the lower temperatures (Bravi *et al.* 2012). The role of lipids in beer has often been associated with adverse effects on beer quality with some long chain fatty acids such as the degradation of linoleic and linolenic acids to trans-2-noneal being associated with off flavours due to the oxidative degradation capabilities (Bravi *et al.* 2014).

Recent research has started to indicate more beneficial roles for lipids, as with other ingredients, at the right concentrations. One such role is in yeast metabolism, where long-

chain unsaturated fatty acids contribute positively to the activation of yeast cell growth under anaerobic conditions, contributing to a quicker fermentation (Bravi *et al.* 2009). Another area where lipids considerably affect beer quality is in foam stability. Beer foam is regarded as one of the most important indicators of beer quality, with specific reference being made to foam stability where lipids play an important role due to their hydrophobic interactions and the foam stability can be affected by fatty acid profile as well as other factors such as hop acids, proteins, and polysaccharides (Gordon *et al.* 2018). Short chain fatty acids such as C6-C10 do not disrupt any of the characteristics of beer foams. However, as the length of the fatty acid chain increases, the more unstable the beer foam becomes, leading to undesirable sensory properties (Wilde *et al.* 2004). This action is only possible due to the ability of lipids to have hydrophobic and hydrophilic interactions, concentrating at surfaces with the hydrophilic portion in contact with the aqueous phase of beer and the hydrophobic tails interacting with the gas within the bubbles of the foam. This action disrupts protein-protein interactions by displacing proteins as they are absorbed into the liquid-gas interface and it is the competition interaction of these surface-active compounds for places in the bubble walls that contributes to foam destabilisation, increasing the chances of coalescence of these bubbles until the eventual loss of foam over time (Bamforth 1985).

Despite being present in beer in such low concentrations, lipids can have positive and negative effects on various aspects of beer quality depending on the type of lipids present, storage conditions that allow oxidation, and the concentrations present. However, of the approximately 3% w/w lipid content of barley, it has been estimated that lipid content in beer can be less than 0.1% (Buiatti 2008). This is due to the insolubility of many lipids, as evident by their hydrophobic region, the loss of some during malting, and the loss of the remainder to the barley by-products of the brewing process called brewer's spent grain (BSG) (Buiatti 2008). BSG is the most abundant of the by-products accounting for

approximately 85% of the total by-products and is the insoluble remains of the mashing process, following the removal of the liquid portion, the sweet wort, that contains the fermentable sugars for yeast (Mussatto 2014). It is estimated that 20 kg of BSG is produced for every 100 L of brewed beer, and due to its high content of undegradable protein (19-30% w/w), fibre (30-50% w/w), lipids (~10-13% w/w), and water soluble minerals alongside phenolic compounds and it is used as feed for the livestock industry (Buffington 2014; Lynch *et al.* 2016). The composition of brewers spent grain has been analysed with the lipid content being majority triglycerides (~55%), free fatty acids (~30%) and phospholipids accounting for (5-9%) (Niemi *et al.* 2012). The fatty acid profile of BSG contains linoleic (18:2), palmitic (16:0), and oleic acids (18:1) as the most abundant lipids with lesser amounts of stearic and linolenic acid along with the presence of phenolic compounds (Niemi *et al.* 2012; Connolly *et al.* 2013).

These values can vary as the chemical composition of BSG is subject to change due to factors such as malt variety, growing, and harvest conditions and the mashing conditions during brewing (Gupta *et al.* 2010). Interest in the potential use of BSG has grown significantly over the past few years, possibly due to economic and environmental pressures to reduce the waste we produce and find novel uses for it, which has prompted investigations into BSG for its potential to benefit human health, as an ingredient in novel food applications and even for energy production such as thermochemical conversion and biogas production (Mussatto 2014).

Spent hops (SPHP) is another by-product of the brewing industry. These occur as part of the second solid residue produced during brewing following the boiling of the wort where the SPHP along with some other solid residue precipitate such as coagulated proteins that were denatured during boiling (dos Santos Mathias *et al.* 2014). Of the mass of the hops used, 85% is disposed. Due to the bitterness compounds present it is not suitable for animal

feed as it is not eaten, even when combined with the BSG. Though not much attention has been given to the SPHP for potential health benefits, it has been used as a fertiliser or compost for farmers with one investigation using essential oils extracted from SPHP as an eco-friendly repellent for insects that cause loses of stored foods (Bedini *et al.* 2015; Kerby and Vriesekoop 2017).

These by-products are produced at such a high rate not only on an industrial level, but in addition to this there is approximately 63 craft breweries operating in Ireland as of 2016 further creating brewing industry wastes. The term craft brewery is defined as a brewery involved in the small-scale production (less than 30,000hL) of beer and despite producing by-products on a smaller scale than larger industrialised breweries it is still estimated that these microbreweries could produce as much as 241,000hL of beer (Flannery Nagel Environmental Ltd 2016). The fact that so many by-products are produced with significant protein, lipid, fibre, phenolic, and mineral content still within them, the valorisation of these by-products for the benefit of human health is a potential route that could be explored further.

1.8 Beer and Health

It is difficult to write about the potential of alcohol-based products as beneficial aspects of the diet without discussing the negative impacts excessive consumption can have to human health. The harmful effects of alcohol are more known than any beneficial factors due to their implications in a variety of chronic diseases such as cancer and obesity (Temple 2012).

Moderate alcohol consumption, mainly from wine, is a key constituent of the Mediterranean diet and is associated with improved cardiovascular health (Estruch *et al.*

2018). This is normally consumed with a balanced meal and these beneficial effects have been shown in postprandial studies investigating these health effects (Xanthopoulou *et al.* 2017). This has been described as the J-shaped curve, or sometimes 'U-shaped curve' where those who consume 1 or 2 standard drinks per day are at 20-30% reduced CVD risk than those who abstain, which is similar to the observations that led to the term 'French paradox' (Renaud and de Lorgeril 1992; Thompson 2013)

The negative effects of alcohol consumption are more associated with over consumption of alcohol and in cases of alcohol abuse, but it has been shown to have beneficial effects when consumed in moderation with most literature focused on wine due to its role in the Mediterranean diet. Beer, though not studied as heavily as wine, has been associated with several potential health benefits also (de Gaetano *et al.* 2016). Moderate consumption of alcohol refers to approximately 1-3 units of alcohol and intake at this level has been associated with a lower risk of developing CHD (Bamforth 2002; Mathews *et al.* 2015). The mechanisms by which alcohol achieves this lower risk of CHD has been associated to a number of mechanisms, though it has been demonstrated in several studies that alcohol increases the concentrations of serum high-density lipoprotein cholesterol, which helps in slowing or preventing the development of atherosclerotic lesions, while other authors have credited the cardioprotective properties to its ability to slow or prevent the action of PAF during chronic inflammation by competitive displacement or supporting the synthesis of molecules that help balance the serum levels of PAF in the body (Thornton *et al.* 1983; Lordan *et al.* 2019a; Lordan *et al.* 2019b).

In summary, polar lipids from various sources have demonstrated antithrombotic and anti-inflammatory effects against a range of chronic diseases that stem from chronic inflammation. Many of these polar lipids have been extracted from food and beverages that have been observed as components of the Mediterranean diet. Reference was made to wine,

cheeses and yogurts as cardioprotective components of this diet with the process of fermentation being a core process of many of these cardioprotective foods suggesting that fermentation could be a factor in the enhancement of the antithrombotic capabilities of polar lipids from these sources.

Chapter 2:

Materials and methods

2.1 Chemicals and Reagents

The experimental procedures conducted as part of this research were lipid extraction, thin-layer chromatography (TLC), platelet aggregation analysis, and gas chromatography–mass spectrometry (GC-MS) with all procedures being carried out at the University of Limerick. Glassware, solvents, and chemicals were purchased from Fisher Scientific Ireland Ltd. (Dublin, Ireland). All reagents used for platelet aggregation analysis and the lipid standards required for GC-MS and TLC analysis, along with the TLC plates were purchased from Sigma-Aldrich (Wicklow, Ireland). All platelet aggregometry consumables were purchased from Labmedics LLP (Abingdon on Thames, U.K.). All GC-MS consumables were purchased from Apex Scientific Ltd (Kildare, Ireland).

2.2 Production of Beer and Origin of Associated Samples

Munster Brewery (Youghal, Co. Cork, Ireland) provided all the relevant samples for this investigation. The beer, an Irish red ale sold by the brewery under the commercial name '12 Towers' is brewed in accordance with organic certification by the Irish Organic Association using only organically sourced materials. All samples investigated were associated with the brewing of this Irish red ale from a single production line at Munster Brewery with the following controlled parameters.

The malted barley used was composed of two barley malts. Organic pale ale made up ~70% of the overall malt, which was kiln dried at 90-95℃ providing a base malt while the other was 30% was kiln dried to 230℃ yielding a roasting grain. This yields the malted barley, the first of the ingredients investigated in this study. Both have a maximum moisture content of 4.5% and on the European Brewing Convention (EBC) colour scale, the base malt has an EBC colour of between 7 and 10 and the roasting malt an EBC colour between 1,000-1,400 (Castle Malting Ltd., Beloeil, Belgium) contributing to a unique colour, flavour, and

aroma profile in the final product. Only the malted grains and not the raw grains could be sourced for this study and the malting process was determined by the company (Castle Malting Ltd., Beloeil, Belgium), this is standard for microbreweries. This malted barley was milled by the brewery and added to the mash tun where 4L of water was added per kg of crushed grain where the steeping process ran at 66℃ for 60 minutes, facilitating the conversion of starch to simple sugars. The temperature was then raised to 77-79℃ for 4 hours as part of the lautering process to denature the active enzymes responsible for this conversion of starch to sugars. Following this step, the BSG sample was stored, the first of the by-products examined in this study.

The liquid phase (referred to as the sweet wort) remaining in the mash tun was then transferred to the boiling kettle where the wort was heated to 100℃ and left to boil for 1 hour. This ensures the sterilization of the wort, denaturation and precipitation of proteins, and facilitates the caramelisation of some of the sugars present that contribute to the taste and aroma profile of the beer. The second ingredient investigated was the hops. A specific amount of organic hops was added (~ 200g/hL) at the beginning of the boil. The hops contain many volatile compounds (Aberl and Coelhan 2012) that contribute to the sensory properties of the beer. At the end of this process the liquid portion was filtered into the fermentation tank by whirlpooling, which yields the second by-product investigated, the SPHP otherwise known as the trub. The trub is the name given to the remaining hops and residual grain material in the brew kettle after the wort has been filtered into the fermentation tank by whirlpooling. This whirlpooling process passes the wort through heat exchangers that cools the wort to 20℃, at which point the in-process wort sample was taken, before passing it to the fermentation tank.

The fermentation was carried out by the yeast *Saccharomyces cerevisiae* (Nottingham High Performance Ale Yeast, Lallenmand Inc., Burton upon Trent, UK), the

most commonly used top fermenting yeast. The yeast was pitched into the fermentation tank at 80-100g of yeast per hL of wort and the fermentation was let to run for 4 days followed by a conditioning period of 7 days at 12℃, and finally 3-4 days at 0-2℃ in a chilling tank in a process known as cold crashing, which facilitates flocculation, sinking, and removal of the yeast.

Once chilling was complete the beer was bottled in the presence of 2g of 100% fermentable organic dextrose (Charles Faram Ltd., Worcester, UK) to encourage carbonation by any surviving yeast in the beer, this is known as bottle priming. This priming occurs for 14 days at room temperature until the desired CO_2 levels were achieved (2.2-2.5 volumes of CO_2), following which the bottles were refrigerated (0-4℃) until they are sent to the laboratory for testing. The final product is the beer analysed in this study. Samples were obtained in triplicate from the highlighted points during different batches of beer production in air-tight containers and transported to the University of Limerick where they were frozen on arrival for preservation for a maximum of six weeks.

2.3 Extraction

The lipids were extracted from the brewing materials, by-products, wort, and beer from three different batches of beer from the same production line.

The TL from all samples were extracted in triplicate using the Bligh and Dyer (1959) method, which is a method used for the efficient extraction of lipids from a biological material (Bligh and Dyer 1959). The TL extraction was achieved by homogenisation of the sample in chloroform/methanol/water in a 1:2:0.8 (v/v/v) ratio followed by filtration into a separatory funnel. Once filtered the chloroform/methanol/water ratio was adjusted to 1/1/0.9 (v/v/v) to achieve phase separation with the TL being present in the lower phase. This phase

was gathered in a round-bottom flask and evaporated until dry on a rotary evaporator at 40°C under vacuum between 700-50 mbar (Buchi Rotavapor, Mason Technology Ltd., Dublin, Ireland).

Once obtained, a tenth of the TL was stored under nitrogen at -20°C. The remaining lipid was subjected to a modified method of counter-current distribution of Galanos and Kapoulas (1962) whereby pre-equilibrated petroleum ether and 87% ethanol were used to obtain the TNL and the TPL extracts, with completion of this method yielding the TPL in the ethanol phase and the TNL in the petroleum ether phase within a separatory funnel (Galanos and Kapoulas 1962; Karantonis *et al.* 2008). This modified method, illustrated in Figure 2.1, involved dissolving the TL in 10 mL of 87% ethanol. Then, 2 mL of this solution plus 8 mL of 87% ethanol was washed through the first separatory funnel containing 30 mL of petroleum ether. Phase separation occurs, and the lower phase was extracted into the second separatory funnel containing 30 mL of pre-equilibrated petroleum ether to ensure the separation of the TNL from the TPL. These steps were repeated several times until all sample has been washed through leaving the TPL and TNL in the 87% ethanol and petroleum ether phases, respectively. Both phases were collected in round-bottom flasks and evaporated using a rotary evaporator until dry. The samples were weighed, and all extracts were stored under nitrogen at -20°C until required for further analysis as previously described by Tsoupras et al (2018).

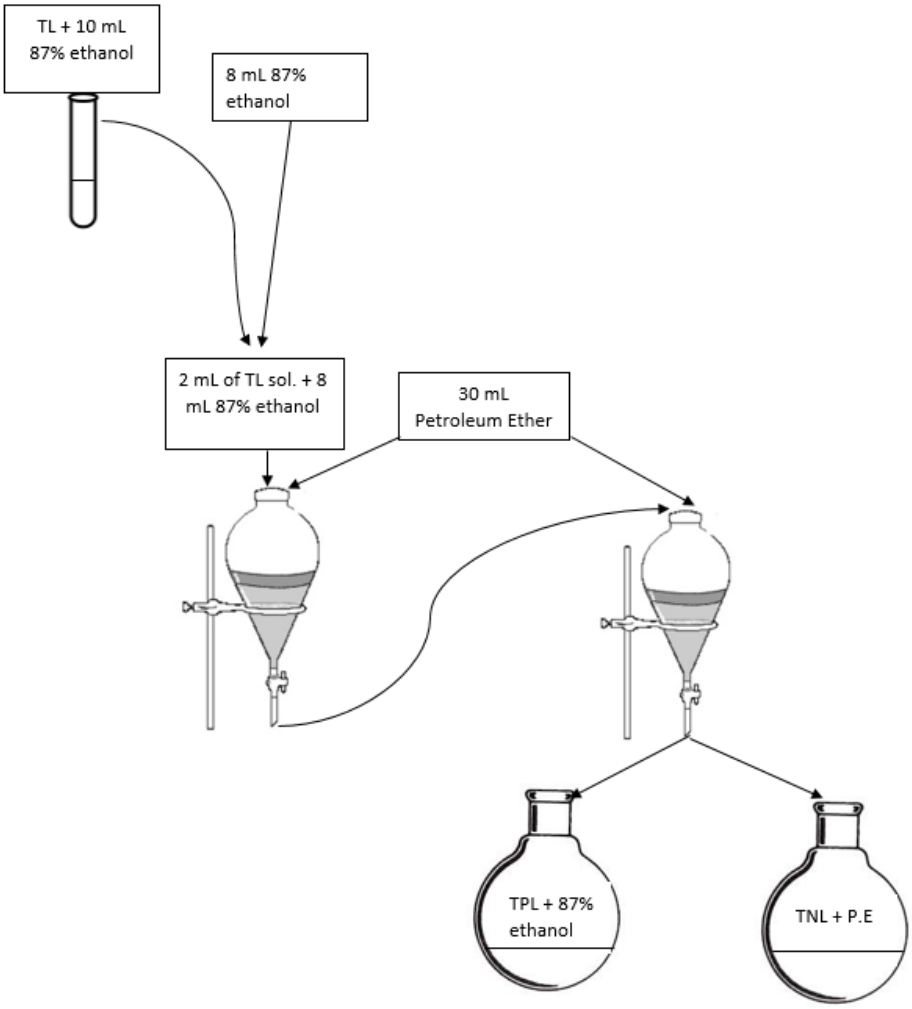

Figure 6.1. Diagram demonstrating the modified counter-current distribution method using increased volumes.

2 mL of the TL in 87% ethanol solution plus 8 mL of 87% ethanol was added to the first separatory funnel. After each addition to the separatory funnel, the funnel was shaken well to ensure the maximum interaction of lipids with the solvents. Once phase separation occurs, the lower phase was transferred to the second separatory funnel, shaken well and the lower phase containing the TPL was extracted to a round-bottom flask. This was repeated until all the TL had been washed through leaving the TNL in both separatory funnels dissolved in petroleum ether (P.E) which was extracted to a separate round-bottom flask**.**

2.4 Thin-Layer Chromatography (TLC)

Phospholipid fractions were obtained by TLC from the TPL of the beer in accordance with previously described methods (Nasopoulou *et al.* 2007; Lordan *et al.* 2019a). To begin, 30 mg of dried TPL from the beer was weighed using an analytical mass balance (Sartorious Ltd.) and diluted in 100 µL of 1:1 chloroform methanol and applied using a capillary tube

to the TLC plate evenly along the bottom of the silica plate. Along the same level, but separated from the beer TPL, a phospholipid standard from eggs purchased from Sigma-Aldrich (Wicklow, Ireland), against which the fractionated bands were compared by their retention factors (R_f). Once the sample was placed on the TLC plate, the plate was placed in an elution tank containing the mobile phase of chloroform, methanol, and water in the ratio of 65:35:6 (v/v/v). As capillary action caused the mobile phase to move up the TLC plate, fractions of phospholipids were eluted from the solution at different times dependant on their affinity to the stationary phase or mobile phase caused by their polarity. Once the solvent front had reached the end point, as seen by the uppermost pencil line on the plate, as seen in Figure 9, the TLC plate was removed and from the elution tank and placed in the fume hood to dry, allowing the evaporation of all solvents before staining. Phospholipids were stained using iodine vapours for visualisation of individual lipid fractions on the plate, the distance travelled by each fraction was marked and compared to the egg standard for identification. Once iodine vapours had evaporated, lipid fractions were scraped from the plate and extracted from the silica gel using the Bligh & Dyer (1959) method. The chloroform phase of the extraction containing the isolated polar lipids were evaporated to dryness under nitrogen, weighed as previously described, and re-dissolved in chloroform:methanol 1:1 (v/v) and stored in sealed vials in the presence of nitrogen at -20°C and stored for a maximum of six weeks, until required for further analysis.

2.5 Aggregometry

The *in vitro* assessment of PAF-induced platelet aggregation was carried out as described in several publications (Nasopoulou *et al.* 2007; Lordan *et al.* 2019a; Fragopoulou *et al* 2000). The protocol was performed in accordance with Declaration of Helsinki, following approval of the protocol by the Ethics Committee of the University of Limerick.

An overnight fasting blood sample was collected from healthy volunteers (n=12) that were not undergoing any form of antiplatelet treatment. The donors gave written consent and were fully aware of the designated use of the blood samples during the study. The blood was withdrawn via venepuncture of the median cubital vein using a technique known as the aspiration method by a clinical nurse using a 20G needle into evacuated sodium citrate S-monovettes (0.106mol/l in a 1:10 ratio of citrate to blood; Sarstedt Ltd., Wexford, Ireland) where the sodium citrate acts as an anticoagulant (Lordan *et al.* 2019c).

The S-monovettes were subjected to centrifugation at 18 x g for 18 minutes at 24°C (Eppendorf 5702 R, Eppendorf Ltd, Stevenage, UK) and the platelet-rich plasma (PRP) was removed. This was followed by a subsequent centrifugation at 1,500 x g for 20 minutes at 20°C. No brake was applied to either centrifugation to prevent platelet activation. The first centrifugation contains the PRP, which formed at the top layer of the S-monovette. This PRP was transferred to a polypropylene tube to be used during the aggregation bioassay while the second centrifugation of the S-monovette allows the isolation of the platelet-poor plasma (PPP) that was used as a blank. The PRP was standardised to 500,000 platelets μL^{-1} by diluting the PRP with PPP until an absorbance of 0.8 was achieved at 530 nm using a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan) prior to analysis on a Chronolog-490 two channel platelet aggregometer (Havertown, PA, USA), coupled to the AGGRO/LINK software package.

Lipid samples (TL, TPL, and TNL) and PAF were prepared prior to testing, dissolving them separately in a solution of Bovine serum albumin (BSA)-saline (2.5mg BSA/mL saline). The PAF concentration in the cuvette was 2.6×10^{-8}. The lipid samples were prepared by dissolving them in known concentrations of BSA (2.5mg BSA/mL saline). Various concentrations of the sample can be added to the platelet bioassay via the following method to assess the sample ability to inhibit PAF-induced platelet aggregation.

Once all samples were prepared, 250 µL of PRP was added to an aggregometer cuvette at 37°C with stirring at 1,000 rpm and was calibrated using the PPP as a blank to represent 0% platelet aggregation. PAF was then added to the cuvette, inducing aggregation of platelets in order to calculate the maximum reversible aggregation (~2.6x 10^{-8} M, final concentration in cuvette) and 50% PAF-induced aggregation. The PAF-induced aggregation was calculated at a range of PAF concentrations to obtain a linear curve with this IC_{50} value being calculated as described by Tsoupras *et al* (2018a). The IC_{50} value is the concentration of sample that induces 50% of PAF-induced platelet aggregation. The PAF curve is used to calculate this IC_{50} value in triplicate (n=3) with a different healthy blood donor for all lipid samples along with the TPL fractions obtained by TLC and expressed as a mean value of the mass of lipid (µg) in the cuvette ± standard deviation (SD). Different concentrations of lipid samples (measured in µg) were added to the platelet suspension and the ability of each to inhibit PAF-induced aggregation was recorded. All samples were tested on the blood within 3 hours of venepuncture.

2.6 Gas Chromatography-Mass spectrometry (GC-MS)

Using approximately 35mg of TPL, fatty acid methyl esters (FAME) were prepared for the beer, wort, brewing ingredients, and by-products similarly to that of the preparation of Tsoupras *et al* (2018a). The FAME method was employed for the rapid derivatisation of fatty acid methyl esters using a 90% solution of 0.5M KOH CH_3OH and subsequent extraction using hexane. To do this, 1 mL of n-hexane and 4 mL of the 90% KOH solution were added to a test tube, vortexed well and, using hydrochloric acid (HCL) and the KOH solution, adjusted to pH 7. At pH 7, phase separation occurs with the addition of 2 mL of H_2O and 2 mL of n-hexane to the solution. The fatty acids orientate to the upper phase, which is then extracted into another clean test tube using a glass Pasteur pipette. Once again

2 mL of n-hexane is added to the original test tube, vortexed, and the upper phase is again transferred to the second test tube. The FAME is extracted following the addition of 2 mL of H_2O to the second test tube and rejection of the lower phase. The obtained fatty acid methyl esters were dried under nitrogen and weighed before being dissolved in 90 µL of hexane and 10 µL of the 21:0 (2000ppm) internal standard.

The GC-MS analysis was carried out similarly to that described by Tsoupras *et al* (2018a), by using the internal standard method and was conducted using an Agilent J&W DB-23 fused silica capillary column (60 m, '0.25 mm i.d.' 0.25 µm f.t.; Agilent Technologies Ltd., Santa Clara, CA, USA). A five-point calibration curve was prepared using five solutions of heptadecanoic acid methyl esters (17:0 at 50ppm, 100ppm, 200ppm, 400ppm, and 800ppm) and five 500ppm injections of heneicosanoic acid (21:0) methyl esters on a Varian 410-Gas Chromatographer coupled to a Varian 210-MS detector equipped with a split/splitless injector (Agilent Technologies, Pal Alto, CA, USA). The Calibration curve describes the line as $y = 0.0041x + 0.12$ with an R^2 of 0.9969, visible in the appendices as Figure 8, where the ratio of 17:0 to 21:0 is represented by the y-axis variable, while the concentration (ppm) of 17:0 represents the x-axis variable of the calibration curve. Using 1 µL injections, this equation was used to find the concentrations of fatty acids within the lipid sample where the ratio of the area of the analyte peak to that of the internal standard peak is this time representing the y-axis value, while the x-axis value represents the analyte concentration of the fatty acid associated with the peak selected.

Injection of 1 µL into the Varian 410-GC set at 230°C with a split ratio of 1:20 begins the separation process. The flow rate of the carrier gas, high purity helium, was set to 1 mL/min, the oven temperature was initially set to 100°C for 5 minutes, rising to 240°C at a rate of 3°C/min and finally isothermal at 240°C for 10 minutes. The retention time, mass spectra, and peak area of the fatty acids were obtained. These values were used to identify

the FAME when they were compared to a pre-derivatised 37-component FAME standards mix (Sigma Aldrich, Wicklow, Ireland), which allowed for identification of fatty acids within the analyte by comparison of their retention times and mass spectra of these peaks. Varian Star Chromatography Workstation Version 6 software (Aglient Technologies, Palo Alto, CA, USA) and a NIST library of mass spectra (Gaithersburg, MD, USA) was also used to aid the identification of fatty acids.

2.7 Statistical Analysis

All experiments were completed in triplicate with the obtained results expressed as a mean value ± standard deviation (SD). These values were obtained using one-way analysis of variance (ANOVA) and the Kruskal-Wallis non-parametric test to determine significant statistical differences between analyses if present for all data except for the analyses of the GC-MS data where one-way analysis of variance (ANOVA) and the Tukey's honest significant difference (HSD) multiple comparison post-hoc test was used (SPSS Inc., Chicago, 215 IL, USA).

Chapter 3:

Results

3.1 Extraction

The TL was extracted in triplicate (n=3) from three separate samples of the beer, wort, and the grain and hop ingredients and by-products. The TL of these samples were separated to TPL and TNL. These results are displayed on Table 3.1 expressed as g of lipids per 100g of sample, with the TPL and TNL also being displayed as a percentage of the TL.

Table 3.1. The Total lipid (TL), total neutral lipid (TNL) and total polar lipid (TPL) expressed as g/100g of sample with TNL and TPL also being expressed as a percentage of TL (mean ± SD, n=3)

	TL (g/100g)	TNL (g/100g)	TNL (% TL)	TPL (g/100g)	TPL (% TL)
MG	0.70 ± 0.10^{a}	1.03 ± 0.03^{a}	13.6 ± 2.9^{c}	0.52 ± 0.05^{a}	74.5 ± 3.5^{b}
BSG	1.05 ± 0.19^{a}	0.55 ± 0.11^{b}	52.6 ± 2.5^{e}	0.41 ± 0.09^{a}	38.9 ± 2.3^{a}
RWHP	14.17 ± 2.18^{b}	1.63 ± 0.31^{c}	11.5 ± 3.7^{bc}	11.6 ± 1.68^{b}	79.4 ± 8.6^{b}
SPHP	0.75 ± 0.06^{a}	0.16 ± 0.01^{a}	21.4 ± 3.0^{d}	0.55 ± 0.07^{a}	72.6 ± 4.1^{b}
Wort	0.03 ± 0.002^{a}	0.002 ± 0.001^{a}	5.4 ± 1.6^{ab}	0.03 ± 0.001^{a}	84.5 ± 8.9^{bc}
Beer	0.02 ± 0.004^{a}	0.0003 ± 0.0001^{a}	1.7 ± 0.4^{a}	0.02 ± 0.005^{a}	91.3 ± 2.7^{c}

^{a,b,c,d,e} are superscripts used to indicate significant differences among different lipid extracts within the same lipid class ($p < 0.05$) when samples are compared using Kruskal-Wallis non-parametric test. Abbreviations: MG = malted grain; BSG = brewer's spent grain; RWHP = raw hops; SPHP = spent hops.

The amount of TL obtained from the MG and the BSG was low and it can be seen that the TPL accounts for a large portion of the TL present within the MG. It was observed that the TNL makes up a larger proportion of the BSG than the MG. The raw hop (RWHP)

sample has a significantly higher TL than all other samples with a large proportion of this being TPL. After boiling, a significant reduction can be seen in the quantity of TL in the SPHP from the RWHP though the relative amount of TPL in the TL is still similar to the RWHP. The quantity of lipids extracted from the ingredients into the wort and beer is significantly lower with TPL accounting for large proportions of the TL as is in accordance with the literature (Bravi *et al.* 2014; Lordan *et al.* 2019a).

3.2 Aggregometry

The antithrombotic activity of the TL, TNL, and TPL of samples are presented in Table 3.2**.** Each sample was measured for their ability to inhibit PAF-induced platelet aggregation *in vitro* with the results being presented as the IC_{50}, a measure of the mass of lipid, in micrograms (µg), that is required to induce half maximal-reversible aggregation via interactions with PAF or PAF-R in the PRP, meaning a lower value IC_{50} is an indication of high inhibitory action against PAF-induced human platelet aggregation. The TL of the MG, RWHP, and SPHP showed moderate levels of inhibitory activity against PAF while the TL of the BSG, wort, and beer showed high levels of inhibition against PAF-induced platelet aggregation. In many cases, the biological activity of the TL could be attributed to the action of the TPL. However, in the BSG the TL displayed potent inhibitory action against PAF despite the TNL and TPL displaying much lower biological activity. This is unusual, but this could be attributed to synergistic action of the TPL and TNL of the BSG. However, further research is required.

The inhibition of the TPL and TNL extracted from the TL also displayed inhibitory action against PAF-induced platelet aggregation. Of the TNL examined in the bioassay, MG, wort and beer were the only extracts that exhibited any significant biological activity towards PAF with moderate levels of inhibition. Moderate-low biological activity was induced by the TPL of the samples tested with the exception of wort and beer. The wort

displayed a strong inhibitory action towards PAF displaying a low IC_{50}. The TPL of the beer displayed the most inhibitory action against PAF-induced platelet aggregation with a very low IC_{50} result being observed which is in accordance with literature (Lordan *et al.* 2019a). The increase in biological activity between the wort and beer TPL highlights that fermentation affects the biological activity of the lipids, which was statistically significant ($p < 0.05$) when the wort and beer were compared using Kruskal-Wallis non-parametric test.

Table 3.2. The *in vitro* antithrombotic properties of the TL, TNL and TPL of all samples against PAF-induced platelet aggregation. The results are presented as mean IC_{50} value in micrograms (µg) ± SD (n=3).

	TL	**TNL**	**TPL**
MG	495 ± 105^{c}	298 ± 89^{a}	191 ± 58^{c}
BSG	69 ± 33^{b}	610 ± 136^{c}	617 ± 184^{d}
RWHP	453 ± 109^{c}	1088 ± 172^{c}	473 ± 280^{cd}
SPHP	519 ± 81^{b}	924 ± 166^{bc}	436 ± 142^{cd}
Wort	70 ± 29^{b}	175 ± 61^{a}	58 ± 11^{b}
Beer	6.4 ± 4.5^{a}	248 ± 66^{a}	7.8 ± 3.9^{a}

[a,b,c] Different subscripts indicate significant differences among different lipid extracts within the same lipid class ($p < 0.05$), when samples are compared using Kruskal-Wallis non-parametric test. Abbreviations: MG = malted grain; BSG = brewer's spent grain; RWHP = raw hops; SPHP = spent hops.

The TPL of beer was fractionated by TLC, the isolated bands scrapped, extracted by a modified method of Bligh & Dyer (1959), dried, and the mass was determined using an analytical mass balance (Sartorius UK Ltd., Epsom, UK). These fractions were then subjected to examination in the bioassay. Bands 1 and 7 displayed poor inhibition against PAF. Some moderate antithrombotic activity was shown by bands 4 and 5. A low IC_{50} value was displayed by band 6 showing potent PAF-inhibition, though it was bands 2 and 3 that displayed very potent biological activity against PAF-induced platelet aggregation with very low IC_{50} values as can be seen in Table 3.3. Figure 3.1 displays the antithrombotic activity of the isolated bands from the beer TPL relative to each other, highlighting the potent antithrombotic nature of the lipid fraction isolated from bands 2 and 3.

Table 3.3. The *in vitro* antithrombotic properties of Beer TPL lipid fractions from beer against PAF-induced platelet aggregation. R**e**sults are presented as mean IC_{50} value in micrograms (µg) ± SD (n=3)**.**

	Band 1	**Band 2**	**Band 3**	**Band 4**	**Band 5**	**Band 6**	**Band 7**
IC_{50} (µg)	605.7 ± 96.7	2.2 ± 0.9	2.7 ± 2.2	110.8 ± 18.9	114.4 ± 46.7	41.6 ± 20.9	302.0 ± 49.9

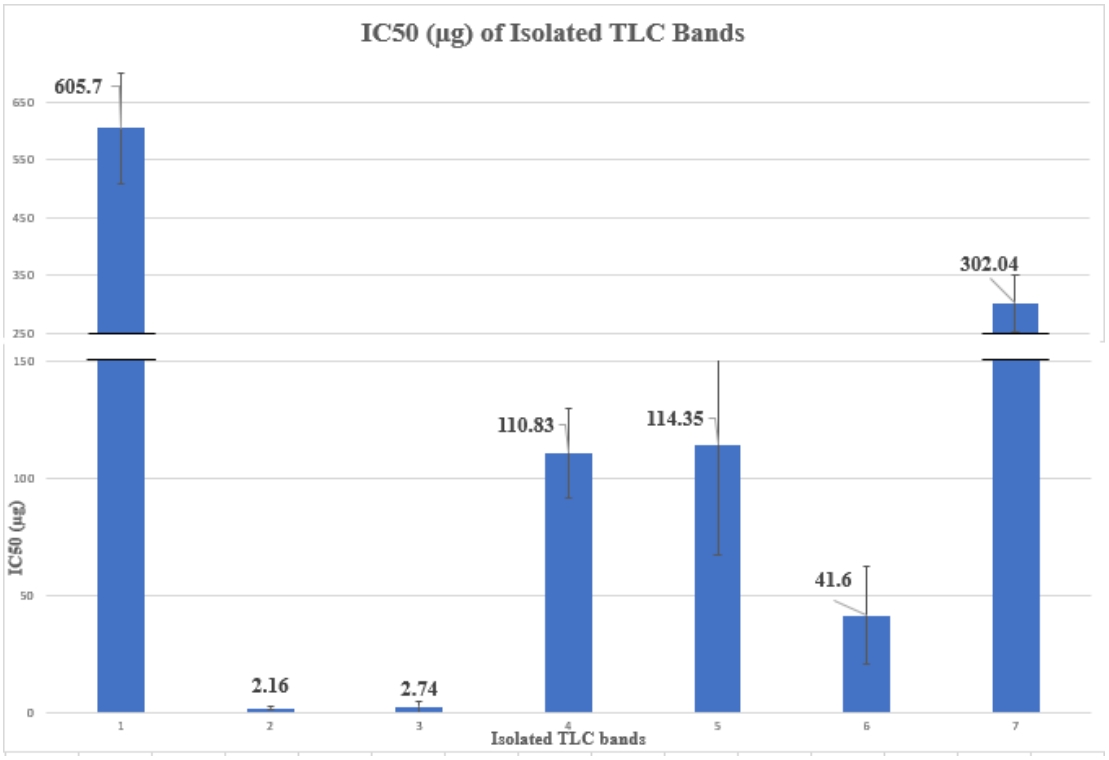

Figure 7.1. The mean IC_{50} (µg) values for the TPL bands of the beer isolated using TLC with error bars of standard deviation (mean ± SD, n = 3).

A photograph of the stained TLC plate prior to the isolation of fractionated bands is visible in the appendices, Figure 9. The beer sample was placed in the middle lane and the egg standard was placed on both outermost lanes for reference.

3.3 Thin-Layer Chromatography

Using TLC, the beer TPL was split into their phospholipid subclasses and the R_f values were obtained. This was done by measuring the distance travelled by the phospholipid fraction and dividing it by the distance by the solvent front. The R_f values of the beer were obtained and compared with the R_f values of the standard which are L-PC, SM, PC, PE and CL. R_f values similar to that of the egg standard were recorded along with two unidentified bands. PC and PE were the most visible on the TLC plate indicating a higher presence of unsaturated fatty acids in these bands. (Arranz *et al.* 2012; Lordan *et al.* 2019a). Seven bands were scrapped from the plate and isolated for testing in the bioassay, the results of which are

displayed in Table 3.3 as IC_{50} (µg) values. The TLC plate prior to the removal of the fractionated TPL can be seen in the appendices as Figure 9.

3.4 GC-MS

The fatty acid composition of the TPL of all samples was investigated and the results are displayed in Table 3.4. The fatty acid profile displayed in Table 3.4 though these fatty acid profiles are associated with the brewing of this particular red ale. As mentioned, growing conditions and variety of the barley, method of hop addition and fermentation variables can all alter the fatty acid profile, which can cause different beers to have different fatty acid profiles which has previously been shown in 3 beer varieties (Lordan *et al.* 2019a).

The most abundant fatty acids found in the TPL were 18:3, 18:2, 18:1, 18:0, and 16:0 which is similar to that what is known of the fatty acid profile of the lipid composition beer, wort and barley in general (Evans *et al.* 2013). Some significant changes can be observed between the ingredients and their associated by-products. Statistically significant differences were observed in the percentage of some fatty acids between samples in the TPL. In the MG and BSG, there was a significant rise in the percentage of fatty acids such as 16:0, and 18:1 t13 but a reduction in the percentages of 18:1 c9, 18:2 along with the absence of some short chain fatty acids in the BSG that were detected in low amounts in the MG such as 22:0, 22:1 and 22:6.

In the SPHP, a statistically significant rise was observed when compared to the RWHP sample in the percentage of numerous fatty acids, but the most notable was a rise in the percentage of 16:0, 18:1 c9, and 18:2 c9 c12. Some of the large changes observed could be associated with the potential presence of proteins and other by-products that are precipitated and removed during the boiling. Detection of several phytochemicals was also observed, listed in Table 3.4, with the largest proportions being observed in the RWHP with low

percentages of phytochemicals being observed in the final product. Of the additional volatiles detected in the beer, 1.1% was attributed to hexanedioic acid (also known as adipic acid E355). The exact source of the adipic acid is unknown as it was not an intentional additive to the product.

The fatty acid profile of the TPL of the beer and wort was also examined with most of the statistical difference in the fatty acids occurring from reduction in the percentages of 18:0, 18:1 c9, 18:1 t13, 18:2 c9 c12 and 18:3 c9 c12 c15 in the TPL. These reductions were accompanied by a significant increase in the percentage of 20:4 present in the beer despite this fatty acid not being detectable in the grain or hops. The changes in the fatty acid composition of the wort and beer are noteworthy when considering that fermentation is the only process that happens between these two samples in the brewing process, highlighting further the potential effect of fermentation on the fatty acid profile.

Table 3.4. Fatty acid and phytochemical composition of brewing ingredients and by-products, wort, and beer. Results are presented as percentage of detected volatile compounds (mean ± SD, n=3)

Fatty acids	Malted Grain	Brewer's Spent Grain	Raw Hops	Spent Hops	Wort	Beer
8:0	ND	ND	0.035 ± 0.009^{b}	0.023 ± 0.007^{ab}	0.013 ± 0.004^{a}	ND
10:0	ND	ND	0.055 ± 0.003^{b}	ND	0.013 ± 0.004^{a}	0.017 ± 0.005^{a}
12:0	0.165 ± 0.016^{d}	ND	0.023 ± 0.002^{a}	0.026 ± 0.006^{a}	0.090 ± 0.009^{c}	0.068 ± 0.003^{b}
12:1	ND	ND	0.048 ± 0.006	ND	ND	ND
14:0	0.615 ± 0.059^{b}	0.688 ± 0.064^{b}	0.334 ± 0.053^{a}	1.043 ± 0.106^{c}	1.584 ± 0.040^{d}	1.547 ± 0.094^{d}
14:1	ND	0.412 ± 0.114	ND	ND	ND	ND
15:0	0.309 ± 0.036^{a}	0.224 ± 0.072^{a}	0.493 ± 0.138^{b}	0.249 ± 0.010^{a}	0.169 ± 0.015^{a}	0.160 ± 0.018^{a}
16:0	19.83 ± 0.928^{a}	27.86 ± 0.802^{b}	22.05 ± 1.230^{a}	30.38 ± 0.955^{c}	31.80 ± 0.599^{c}	32.34 ± 0.683^{c}
16:1	0.385 ± 0.037^{a}	0.295 ± 0.120^{a}	2.354 ± 0.295^{c}	1.147 ± 0.153^{b}	0.604 ± 0.015^{a}	0.333 ± 0.205^{a}
17:0	0.176 ± 0.015^{a}	ND	1.226 ± 0.032^{d}	0.456 ± 0.031^{c}	0.241 ± 0.019^{b}	0.226 ± 0.013^{ab}
17:1	0.087 ± 0.014^{a}	ND	0.746 ± 0.035^{d}	0.356 ± 0.026^{c}	0.203 ± 0.014^{b}	0.203 ± 0.034^{b}
18:0	2.628 ± 0.621^{ab}	2.231 ± 0.250^{a}	2.846 ± 0.083^{abc}	3.854 ± 0.175^{d}	3.610 ± 0.091^{cd}	3.324 ± 0.103^{bcd}
18:1 c9	9.042 ± 0.186^{d}	8.813 ± 0.440^{d}	4.387 ± 0.106^{a}	6.670 ± 0.257^{d}	6.118 ± 0.242^{bc}	5.617 ± 0.179^{b}
18:1 t13	0.657 ± 0.024^{a}	1.009 ± 0.093^{ab}	1.271 ± 0.159^{bc}	1.815 ± 0.242^{d}	1.415 ± 0.089^{c}	1.263 ± 0.136^{bc}
18:2 c9 c12	56.67 ± 0.770^{e}	51.83 ± 1.592^{d}	25.46 ± 1.471^{a}	40.68 ± 0.344^{b}	44.78 ± 0.063^{c}	43.38 ± 1.554^{bc}

18:3 c6 c9 c12	ND	ND	0.575 ± 0.016^{b}	0.117 ± 0.012^{a}	ND	ND
18:3 c9 c12 c15	6.802 ± 0.954^{abc}	5.856 ± 0.540^{ab}	23.42 ± 1.433^{d}	8.829 ± 0.918^{c}	7.720 ± 0.712^{bc}	5.132 ± 0.256^{a}
20:0	0.583 ± 0.103^{ab}	0.781 ± 0.172^{bc}	1.022 ± 0.042^{c}	0.482 ± 0.069^{a}	ND	ND
20:1 c13	ND	ND	0.254 ± 0.054^{a}	0.538 ± 0.035^{c}	0.386 ± 0.019^{b}	ND
20:2 c11 c14	ND	ND	1.002 ± 0.052^{c}	0.493 ± 0.073^{b}	0.268 ± 0.014^{a}	0.306 ± 0.038^{a}
20:4 c5 c8 c11 c14	ND	ND	ND	ND	0.519 ± 0.056	4.929 ± 0.019
20:5 c5 c8 c11 c14 c17	ND	ND	0.527 ± 0.009^{b}	0.164 ± 0.027^{a}	0.408 ± 0.100^{b}	ND
22:0	0.295 ± 0.0037^{a}	ND	1.317 ± 0.315^{c}	0.719 ± 0.127^{b}	0.413 ± 0.019^{ab}	0.288 ± 0.067^{a}
22:1	0.318 ± 0.085^{a}	ND	0.410 ± 0.057^{a}	0.335 ± 0.089^{a}	ND	ND
22:6 c4 c7 c10 c13 c16 c19	0.469 0.087^{a}	ND	1.460 0.169^{b}	0.433 0.129^{a}	ND	ND
∑SFA	24.43 0.704^{a}	31.79 0.940^{b}	29.40 1.324^{b}	37.23 0.909^{c}	37.94 0.599^{c}	37.97 0.544^{c}
∑MUFA	10.67 0.141^{c}	10.53 0.411^{c}	9.469 0.483^{b}	10.86 0.302^{c}	9.133 0.209^{b}	7.416 0.448^{a}
∑PUFA	63.95 1.53^{c}	57.69 1.090^{b}	52.87 2.657^{a}	50.71 0.673^{a}	53.69 0.589^{a}	53.75 1.761^{a}
Phytochemicals						
Hexanedioic acid	ND	ND	ND	ND	0.277 0.042	1.128 0.208
Aromadendrene oxide	ND	ND	1.769 0.391	0.105 0.018	ND	ND
2,4-Di-*tert*-butylphenol	ND	ND	ND	ND	ND	0.120 0.012
B-Carophyllene	ND	ND	2.020 0.371^{b}	0.365 0.047^{a}	ND	0.069 0.006^{a}
2-Dodecanone	ND	ND	0.066 0.048	0.042 0.008	ND	ND

Cubenol	ND	ND	0.237 0.177	ND	ND	ND
Tau-Cadinol	ND	ND	0.142 0.076	ND	ND	ND
Tau-Muurolol	ND	ND	0.293 0.024	ND	ND	0.075 0.003
∑**Phytochemicals**	ND	ND	8.901 0.319[b]	0.9490.322[a]	ND	1.374 0.217[a]

[a,b,c,d,e] are subscripts used to indicate significant differences between lipid compositions. Different letters in the same row indicate statistically significant difference between the same compound in different samples when mean values ± SD (n=3) are compared using Tukey's HSD multiple comparison test ($p < 0.05$). Abbreviations: MUFA = monounsaturated fatty acids; ND = non-detectable; PUFA= polyunsaturated fatty acids; SFA= saturated fatty acids

Chapter 4:

Discussion

4.1 Introduction to discussion

CVD will remain the top global cause of death if measures are not taken to prevent its development. One such measure is developments in the wealth of knowledge of food and beverages that are natural sources of cardioprotective compounds. The anti-inflammatory properties polar lipids have been explored in several sources including fish (Nasopoulou *et al.* 2011), olive oil and pomace (Karantonis *et al.* 2008), dairy products (Lordan and Zabetakis 2017), and fermented beverages such as wine (Fragopoulou *et al.* 2001) and beer (de Gaetano *et al.* 2016; Lordan *et al.* 2019a). It has also been shown that phenolic compounds can co-migrate with polar lipids during extraction, as has been demonstrated by the presence of 2,4-Di-*tert*-butylphenol. While it has been shown that polar lipids can inhibit the PAF-induced platelet aggregation *in vitro,* the mechanism for the antithrombotic of phenolic compounds has not yet been discovered (Tsoupras *et al.* 2018b). However, studies that have fractionated the TPL of samples and tested their antithrombotic properties against PAF have helped give some insight into the nature of the interactions of certain phospholipid classes with PAF or the PAF-R (Tsorotioti *et al.* 2014; Lordan *et al.* 2019a). The aim of this study was to investigate the antithrombotic properties of lipid brewing ingredients, by-products, wort, beer and beer TPL lipid fractions. The fatty acid composition of the TPL of these samples was also identified to investigate the impact of the brewing process on their composition.

4.2 Extraction

The TL, TNL and TPL were extracted from the malted grain (*Hordeum vulgare)*, hops (*Humulus lupulus)*, wort and beer for analysis. Previous research has investigated the TPL of 3 types of Irish beer finding ale to possess the most antithrombotic activities against PAF-induced platelet aggregation when compared with stout and lager (Lordan *et al.* 2019a). To

extract the lipids from the samples intact, the method of Bligh & Dyer (1959) was used. While this method may not be the most efficient extraction of the TL, as it does not use harsh acids, heat treatment, or hot alcoholic extractions associated with some other methods (Morrison and Coventry 1985), the Bligh & Dyer (1959) method allows for the rapid extraction of the bioactive lipids, allowing further analysis in subsequent steps with reduced risk of the decomposition of some fatty acids compared to harsher methods that employ high heat.

The lipid content of barley is reported at ~3% (w/w) of dry weight in living tissues, however it is estimated that 30% of these are lost during malting due to the hydrolysis of triglycerides to free fatty acids (Anness 1984; Buiatti 2008). This means that a barley sample prior to malting would be estimated to have TL in the region of 3g/100g and after malting it would be ~2.1g/100. TL extracted from the MG was much lower (0.7g/100g) than these figures but these lipids can be difficult to extract from barley before or during malting. Up to 33% of the lipid content is located within the aleurone layer and starch granules of barley, often not being extracted at lower mashing temperatures and being lost to the BSG (MacLeod and White 1962; Evans *et al.* 2013). Other lipids and fatty acids may form interactions with polysaccharides in the barley leaving them tightly bound to starch in the form of amylose-lipid complexes until it is degraded to fermentable sugars, making them difficult to extract prior to milling and mashing without using the heat or acid intensive extraction (Marion *et al.* 2003; Gordon *et al.* 2018). The weight of the TL extracted from the BSG was higher, though not statistically significant than the MG, though the TPL of BSG displayed a statistically significant lower percentage of TPL (38.9% of TL) than that of the MG (74.5% of TL). This is in accordance with literature as the BSG has been subjected to milling and mashing, during which endogenous lipase activity causes the release of free fatty acids from these triglycerides and phospholipid (Niemi *et al.* 2012).

The RWHP sample demonstrated a TL that was significant from the TL of all other samples by weight (14.7g/100g) as observed in Table 3.1. However, the hops used in the brewing of this red ale were added to the brew kettle in the form of pelleted hops. These pelleted hops are highly concentrated and accounts for the high TL content observed in Table 3.1. The role of hops in brewing is to contribute aroma and bitterness compounds to enhance the sensory profile of the beer and it does this through the release of oils into the wort during the boiling step (Lermusieau and Collin 2004). The boiling step is a very energy intensive process and various measures are taken to reduce costs. An example of this is seen in this brewery by the use of condensed hop pellets as opposed to the air-dried female hop flowers. The fatty acid content of air-dried female hop flowers is ~1-2%, the essential oil content is ~0.5-3% and waxes and steroids can range from trace amounts to 25% (Shinohara *et al* 2000; Almaguer *et al*. 2014). The compounds desired for the product make up a low percentage of the hop flower and many of the oils are volatile compounds and are lost to evaporation during boiling. This leaves only some of the hop oils to make it intact to the wort, while others can still remain in the SPHP as seen in the GC-MS data on Table 3.4. These oils make up 0.5-3% of the dry weight of the hops, but within that over 400 hop aroma components have been identified (Sharpe and Laws 1981; Aberl and Coelhan 2012; Almaguer *et al* 2014), so the usage of hops condensed into pellet form to raise the concentration is a practical move by breweries. As previously mentioned, during boiling the hydration of the condensed hop pellets allows the passing of many of the compounds present in the hops to the wort where many are lost in evaporation and some remain in the wort. This aids in explaining why the TL content of the SPHP (0.75g/100g) was observed to be much lower than that of the RWHP (14.17g/100g), as per Table 3.1.

There were no statistically significant differences observed between TL of the wort (0.03g/100g) and the beer (0.02g/100g), with both samples yielding considerably low

amounts of TL, which is in accordance with the available literature as much of the lipid content of barley is lost to the BSG (Anness 1984; Lordan *et al.* 2019a). The low lipid content could also be attributed to filtration steps employed by some breweries such as whirlpooling and the cold crashing method employed by this brewery. These filtration steps are aimed at the removal of proteins and polyphenol compounds, of barley and hop origin, that can interact with one another to contribute to beer chill haze, a limiting factor to the shelf life of a beer (Loch-Ahring *et al.* 2008). The use of these methods highlights just some of the various methods breweries employ for the clarification of the wort and beer, through which a reduction in the lipid content could also be targeted as lipids are associated with the destabilisation of the beer foam (Wilde *et al.* 2004).

4.3 Aggregometry

The biological activity of the TL, TPL and TNL of all samples was investigated *in vitro* against the action of PAF-induced platelet aggregation in human PRP with the results displayed in Table 3.2, while the same activities were investigated in isolated lipid fractions obtained from the TPL of beer using TLC and are displayed in Table 3.3 and Figure 3.1. All results are expressed as IC_{50} which is described as the mass of lipid in micrograms (µg) that is required to induce half the maximal-reversible aggregation caused by PAF-induced platelet aggregation (Lordan *et al.* 2019a).

The TPL of MG showed moderate-low levels of inhibition. However, the MG TPL performed better than both the TPL and TNL of the BSG. An interesting observation from these results is that the TL of the BSG displayed an IC_{50} that was statistically significantly lower when compared to the TPL and TNL of the same sample. The TPL and TNL of the BSG displayed very poor levels of inhibition though the TL of the BSG displayed strong

inhibitory action against PAF as can be seen in Table 3.2. These results could suggest a synergistic interaction between the TPL and TNL that improves their ability to interact with PAF/PAF-R that each class of lipid cannot achieve independently which has been suggested previously (Lordan *et al.* 2019a) though further investigation is warranted. Therefore, due to the potent biological activity of the BSG TL, further investigation is warranted for the valorisation of the BSG. The valorisation of BSG has been extensively studied and is currently used in energy production and chemical processes (Mussatto 2014), as well as a novel growth medium for yeast (Cooray *et al.* 2017). Furthermore, the presence of cardioprotective components found in the TL of BSG suggests the potential for another use of this by-product in the development of nutraceuticals or functional-foods should they successfully be isolated from the BSG possibly by food-grade extractions (Tsoupras *et al.* 2019a). The use of by-products for nutraceuticals or animal feed to improve its lipid profile has previously been demonstrated by the olive industry (Sioriki *et al* 2016) and highlights the potential for BSG to be used in a similar manner due to its availability and low cost (Lynch *et al* 2016).

The RWHP and SPHP displayed poor antithrombotic activity with the best of these samples being the TL of the RWHP. However, the TPL of the RWHP displayed similar antithrombotic activity to the TL, while the TNL displayed very poor activity against PAF activity, which suggests that the of antithrombotic activity of the RWHP TL can be attributed to the TPL that comprises ~79.4% of the TL, as per Table 3.1. The SPHP samples displayed similarly low biological activity against PAF with the TPL displaying a non-statistically significant lower IC_{50} than the TL. As previously mentioned, these SPHP samples are collected after the boiling step and resultingly the SPHP sample will have lost many of the volatile compounds to evaporation or to the wort resulting in little bioactivity in the hops.

A significant amount of activity was seen in the wort and beer against the action of PAF-induced platelet aggregation with the TL and TPL of both samples showing particularly low IC_{50} values, as per Table 3.2, which is in agreement with literature (Lordan *et al.* 2019a). The Kruskal-Wallis non-parametric test was used for all statistical difference tests of the blood analyses and showed a statistical difference between the beer and wort with a significant increase in the antithrombotic activity of the beer when compared to the wort. This indicates that there is a change in the TPL composition of the wort when it is fermented to beer. This is in accordance with studies that have also observed an increase in the antithrombotic capabilities of lipids after fermentation, suggesting a possible role for fermentation in the biosynthesis or activation of potent antithrombotic compounds (Lordan *et al.* 2019c). The antithrombotic activity of the wort prior to fermentation is still significantly more potent than the ingredients and by-products which may be attributed to the presence of certain phenolic compounds previously observed in beer and wine that have been implicated with protective effects against CVD (Arranz *et al.* 2012).

The TPL of the beer was fractionated by TLC and the antithrombotic activity of isolated bands was investigated against PAF-induced platelet aggregation, with the results displayed in Figure 3.1 and Table 3.3 as previously mentioned. This method of separation was a preparatory method used for the isolation and extraction of bioactive lipids which, for this study, was for further analysis in the bioassay and displayed similar potent antithrombotic properties to literature (Lordan *et al.* 2019a). However, the study conducted by Lordan *et al* (2019) identified 6 separate bands during the extraction as opposed to the 7 bands isolated in this study. This could be explained by the isolation of what was thought to have been two separate bands, but the results obtained for bands 4 and 5 displayed in Figure 3.1 and Table 3.3 suggests both were in fact PC. The exploration of the polar lipids observed in beer should be further investigated with structural analysis, such as ultra-high

performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS), to provide more insight into the structure-function relationship of these fractions.

4.4 Thin-Layer Chromatography (TLC)

TLC allowed for the fractionation of the TPL of beer. The bands that were identified in the egg yolk according to their R_f values as SM, L-PC, PC, PE and CL which is in agreement with the literature (Ternes and Jaekel 2009).

Band 1 did not correspond to any of the R_f values of the egg standard and was therefore classified as an unknown, however this fraction displayed the highest $IC_{50,}$ corresponding to the lowest antithrombic activity than all other phospholipid fraction tested. The activity displayed by this fraction did not warrant further analysis.

The R_f values of bands 2 and 3 corresponded to L-PC and SM on the egg standard. These bands displayed very low IC_{50}'s displaying the strongest antithrombotic activity against PAF. However, analysis of L-PC and SM in the past has demonstrated that these lipids do not display potent anti-PAF activity to the level of PE which, as seen in Table 3.3, was not the case (Antonopoulou and Karantonis 2002; Tsorotioti *et al.* 2014). This suggests that the fractions isolated may not be that of SM and L-PC and could suggest the presence of other lipids or phenolic compounds that have been shown to be present in beer and have inhibitory action towards PAF (Vlachogianni *et al.* 2015b). Structural analysis is required to identify the compounds present at each fraction as the biological activity displayed by these compounds against PAF-induced platelet aggregation warrants further analysis.

During the extraction of the lipid fractions, bands 4 and 5 were extracted separately as it was thought that these could be different fractions, though their similar R_f values and levels of antithrombotic activity demonstrated that these bands 4 and 5 were both part of the

same fraction with R_f values corresponding to PC. These fractions displayed the same biological activity and was statistically significantly higher than the other bands (2,3, and 6) which is in accordance with the literature (Nasopoulou *et al.* 2013a).

The R_f value of Band 6 corresponded to the R_f value of PE in the egg standard. PE has been previously shown to demonstrate strong biological activity against PAF and this is reflected in the results obtained in Table 3.3 and Figure 3.1 (Tsorotioti *et al.* 2014).

The R_f of band 7 corresponds to CL of the egg standard. This band displayed very weak activity against PAF induced platelet-aggregation. Despite displaying a similar R_f value to CL, the inhibitory activity of this band was not in accordance with previous studies, which found that CL is generally a potent antithrombotic fraction against PAF-induced platelet aggregation (Tsoupras *et al.* 2018a). The activity displayed by this band suggests that the phospholipids found here are not that of CL, structural analysis of this band is required to definitively identify this band though the activity displayed does not necessarily warrant further investigation.

TLC is an effective technique to facilitate the fractionation and extraction of polar lipids though it does have certain limitations. Corresponding R_f values of the sample and the standard can indicate the presence of a particular phospholipid, although as can be seen by bands 2 and 3, this is not always the case. Further analysis, such as HPLC, is required to sufficiently identify these compounds.

Despite these limitations, TLC allowed for the fractionation, extraction, and examination in a bioassay of individual phospholipid classes and clearly demonstrates the potent antithrombotic properties of these lipids in the beer against PAF, which accounts for the overall activity of the beer TL and TPL.

4.5 GC-MS

The fatty acid profile for the TPL of brewing ingredients and by-products, wort, and beer were assessed using GC-MS analysis, the fatty acid composition along with some of the phytochemicals detected are displayed in Table 3.4. FAME analysis of these polar lipids revealed a higher percentage of 16:0 18:0, 18:1, 18:2, and 18:3 throughout all samples, which is similar to the distribution of lipids in the TL of barley, wort and, beer which corresponds to the extraction results in Table 3.1, demonstrating that the TPL account for 74.5% of the TL composition (Evans *et al.* 2013). This could further support the possibility that it is these fatty acids, found in both the TPL and the TL in abundance, that could have contributed to the biological activity seen in the TL of some samples. Little is known about the fatty acid composition of the TPL of hops, although the results displayed in Table 3.4 demonstrate a statistically significant increase in the percentage of these fatty acids from RWHP to SPHP with the exception of 18:3, which showed a statistically significant decrease in the RWHP to SPHP and wort to beer. The percentage of 20:4 observed in the beer (5.1%) is similar to that found in TPL of ale in literature (Lordan *et al.* 2019a). Furthermore, Table 3.4 shows that this was a statistically significant increase in percentage of 20:4 when the wort (0.5%) was fermented to beer (4.9%). This observation could further support the viewpoint that yeast during fermentation can stimulate changes in composition through their metabolism. This same observation was made in a study by Lordan *et al* (2019) where the fermentation of ovine milk to yogurt showed the presence of 20:4 in the TPL of yogurts inoculated with different combinations of starter cultures, despite 20:4 not being detected in the ovine milk itself, accompanied with a statistically significant improvement of the antithrombotic activities the yogurts in comparison to the ovine milk (Lordan *et al.* 2019c). Though not all were statistically significant, an increase in the percentage of 16:0 accompanied with a reduction in the percentage 18:0, 18:1, and 18:3 was seen between the

wort and beer and also in the ovine milk and most yogurt samples of the study by Lordan *et al*, again highlighting the changes fermentation can have to the composition and biological function of fatty acids (Lordan *et al.* 2019c). Some similarities can also be drawn when viewing the fatty acid composition of the wort and beer when compared to the classic PAF structure. The typical PAF structure is described as composing of 16:0 (68%), 18:0 (27%) or 18:1 (4%) in the *sn*-1 position, an acetic acid on the *sn*-2 position, and a phosphocholine group on the *sn*-3 position (Demopoulou *et al* 1979; Lordan *et al* 2019c). The fatty acid content of the beer showed the presence of 16:0 in abundance in the wort (31.8%) and beer (32.34%). The analyses detected the presence of 18:0 (c9) in the wort (3.61%) and beer (3.32%). The third fatty acid, 18:1, was also detected in the wort (6.1%) and beer (5.62%). While the fatty acid composition of the most potent antithrombotic TPL samples shows certain similarities to the fatty acid composition of PAF structure, as outlined above, further research is required to determine if these structural similarities have a bearing on the antithrombotic capabilities of the lipid sample.

The presence of some phytochemicals was also detected, as can be seen in Table 3.4, within the RWHP and SPHP samples which is to be expected as hops are a source of α and β-acids for bitterness, essential oils for aroma, and biologically active polyphenols though due to their volatile nature many are lost during boiling (van Opstaele *et al*. 2010; Karabín *et al.* 2016). β-Caryophyllene was found in the highest abundance in the RWHP and SPHP, as per Table 3.4, which is in accordance with literature as this sesquiterpene can be found in abundance in a variety of hop plants (Kaškonas *et al.* 2016). β-caryophyllene has been identified as having anticancer properties by affecting the growth and proliferation of cancer cells, as well as having analgesic and anti-inflammatory properties (Fidyt *et al.* 2016) highlights the potent bioactivity of this compound. Another biologically active phytochemical identified was 2,4-*tert*-butylphenol though it is not identified in any other

samples suggesting it could have been a product of fermentation by the yeast as there is no known use of this compound as an additive. This is a possible explanation for the presence of this phytochemical in the beer as there is evidence that 2,4-di-*tert*-butylphenol can be synthesised by certain varieties of plants but can also be synthesised by *Saccharomyces cerevisiae* (Shinohara *et al* 2000). It is not yet known if this photochemical improved the antithrombotic activity of the beer TPL as it was present in such a low quantity, but evidence has shown that 2,4-di-*tert*-butylphenol has been observed as an antioxidant, antifungal and anti-tumour properties (Varsha *et al.* 2015). Tau-muurolol, a cadinene sesquiterpene that originated in the hops, was the only other naturally present phytochemical found in the beer samples, though not much is known about the bioactivities of this compound (Katsiotis *et al.* 1989).

Chapter 5:
Conclusions

Conclusions

The data obtained in this study demonstrate the potent antithrombotic nature of polar lipids within the TPL of beer and related ingredients and by-products. The analysis of the fatty acid profile of each sample, compared with the ability of these lipids to inhibit PAF-induced platelet aggregation, gave an insight into the changes these lipids undergo during the brewing process and how these steps effect their biological activity. The changes that occur as a direct result of fermentation is one such highlight, with changes being observed in the biological activity and fatty acid profile of wort when fermented to beer. This potent bioactivity was further investigated with the extraction of isolated lipid fractions by preparative TLC which further highlights that the potent bioactivity found in beer could be attributed to low amounts of certain classes of phospholipids. This presents a promising avenue of research, whereby the wort and beer TPL fractions could be isolated and their bioactivity against PAF-induced platelet aggregation can be recorded. Following this, structural analysis should be carried out on these fractions to allow the identification of these potent PAF-inhibitors and asses how the process of fermentation alters these lipids, making them more suited to inhibit the activity of PAF.

The determination of the fatty acid profile of the TPL of each sample allowed for the observation of the changing composition of samples as they went through the brewing process, while the TLC analyses showed that different phospholipid classes are responsible for different levels of activity against PAF-induced platelet aggregation. This analysis also demonstrated the presence of phytochemicals that could potentially contribute to the antithrombotic properties observed against PAF-induced platelet aggregation though further analysis of these phytochemicals is required. As PAF is directly implicated in chronic inflammation, a precursor for many chronic diseases, detailed investigations carried out on the structure and functions of these particularly bioactive lipids could lead to the

development of certain nutraceuticals that slow or inhibit the action of PAF, lowering the levels of inflammation. The BSG forms another potential target for valorisation for nutraceuticals or for the development of functional foods as the bioactivities demonstrated by the TL of the BSG warrant further investigation. The level of inhibition of the TL of the BSG against PAF-induced platelet aggregation, coupled with the vast quantity of this by-product produced annually, makes this by-product of the brewing industry a potential target for improved cardiovascular health.

This study contributes to the data surrounding the effects of fermentation on the improved biological activity of lipids, presents BSG as a potential target to be utilised in combatting the development of CVD and provides new information into the fatty acid composition of the TPL of brewing ingredients, by-products, wort, and beer.

Appendices

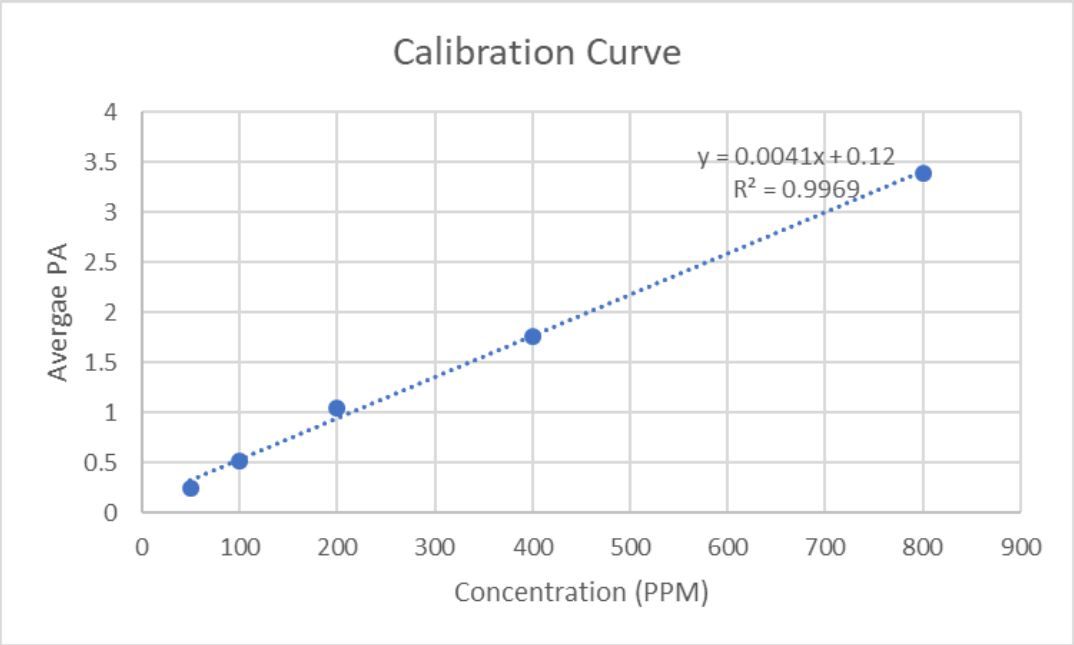

Figure 8. Image displaying the calibration curve used in the GC-MS analyses to calculate the concentration of fatty acids within the TPL samples.

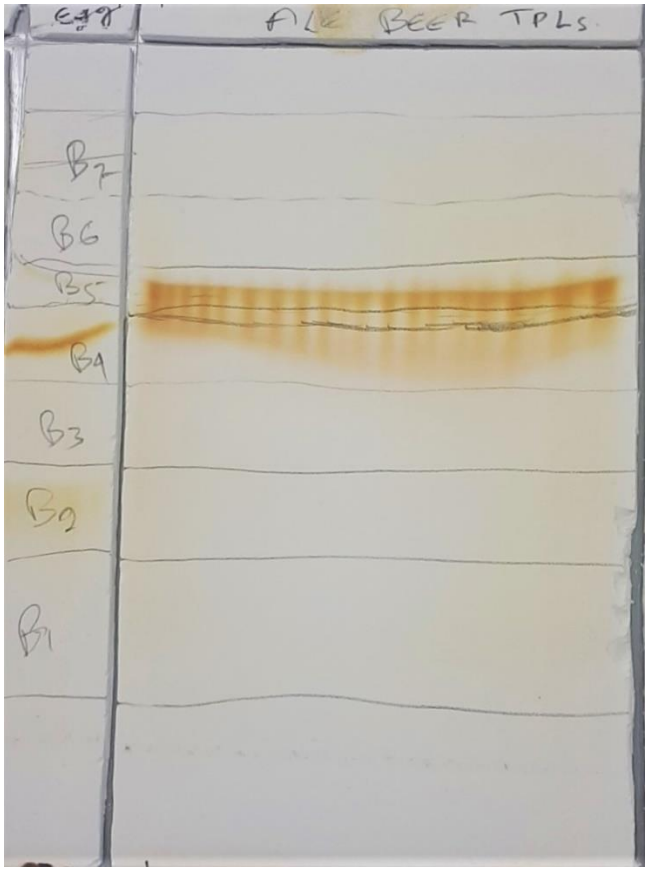

Figure 9. Photograph taken of the TLC plate used for the fractionation of the beer polar lipids.

This image was taken after removal from the visualization tank. The centre column contains the fractionated beer TPL and the column to the left of this is that of the egg standard used.

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